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Enhancement of Blood Levels by Caronamide During Intramuscular Administration of Penicillin. Albert O. Seeler, M.D., Clare Wilcox, and Maxwell Finland, M.D., Boston, Mass. ---------- 807

Enhancement of Plasma Penicillin Concentrations by Caronamide and Sodium Benzoate. Elias Strauss, M.D., Paul L. Richburg, M.D., Phillip Z. Saba, M.D., and J. Ernest Alexander, M.D., Dallas, Texas ---------- 818

A Water-Soluble Preparation for Prolonging Effective Penicillin Levels in Body Fluids. Leo Loewe, M.D., Harold B. Eiber, M.D., Erna Alture-Werber, Ph.D., and Mary Kozak Shore, A.B., Brooklyn, N. Y. ---------- 832

Use of Modified Dubos and Davis Medium for Demonstration of Antibiotic Activity of Subtilin Against Mycobacterium Tuberculosis. Sam C. Wong, Alvin S. Hambly, Jr., and Hamilton H. Anderson, San Francisco, Calif. ---------- 837

Further Observations on the Cultivation of Tubercle Bacilli From Pathologic Material in Dubos Media. George E. Foley, Boston, Mass. ---------- 842

Discrepancies in the Agglutination Test for Brucellosis as Performed With Various Antigens and as Reported From Different Laboratories. C. Wesley Eisele, Norman B. McCullough, and Grace A. Beal, Chicago, Ill. ---------- 847

Human Brucella Melitensis Infections in Minnesota With Hogs as the Probable Source. Paul Kabler, M.D., Ph.D., Henry Bauer, M.S., and C. Barton Nelson, M.D., M.P.H., Minneapolis, Minn. ---------- 854

The Diffraction Method of Measuring Red Blood Cells. Adrianus Pijper, M.D., D.Sc., Pretoria, South Africa ---------- 857

The Role of Phagocytosis in Resistance, as Related to Age of Granulocytes Following Primary and Reinfection Studies With Hemolytic Streptococci in Macacus Rhesus. Samuel Saslaw, Ph.D., M.D., and Charles A. Doan, M.D., Columbus, Ohio ---------- 878

Outbreak of Diarrheal Disease Associated With Paracolon. Herbert F. R. Plass, M.D., Minneapolis, Minn. ---------- 886

(Contents continued on inside front cover)

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The Effect of the Rate of Administration of Amino Acid Preparations and Blood Amino Acid Nitrogen Level on the Production of Nausea and Vomiting. Charley J. Smyth, M.D., Andrew G. Lasichak, M.D., and Stanley Levey, Ph.D., Eloise, Mich. ---------- 889

Study of a New Sedative-Hypnotic Drug (3, 3-Diethyl-2, 4-Dioxotetrahydropyridine). S. Charles Freed, M.D., San Francisco, Calif. ---------- 895

Laboratory Methods

The Assay of Heparin. C. N. Mangieri, B.S., Nutley, N. J. ---------- 901

Permanent Metachromatic Staining of Mucus in Tissue Sections and Smears. Manfred Hess, M.D., and Franklin Hollander, Ph.D., New York, N. Y. ---- 905

Reticulum Staining With Schiff Reagent After Oxidation by Acidified Sodium Periodate. R. D. Lillie, Bethesda, M.D. With the assistance of A. Laskey, J. Greco, and H. Jacquier ---------- 910

The Quantitative Determination of Amphetamine. William D. McNally, A.B., M.D., W. L. Bergman, Ph.C., J. F. Polli, B.S., Chicago, Ill. ---------- 913

A Microscrew Clamp for Use With Mohr Type of Microburettes. R. H. K. Foster, M.D., and A. G. Broeker, St. Louis, Mo. ---------- 918

An Instrument for Recording Multiple Pressures in Man. George S. Edwards, M.D., Los Angeles, Calif. ---------- 921

Books Received

Books Received ---------- 929

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ENHANCEMENT OF BLOOD LEVELS BY CARONAMIDE DURING INTRAMUSCULAR ADMINISTRATION OF PENICILLIN

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THE rapidity with which penicillin disappears from the blood and is excreted in the urine was recognized early in the study of this antibiotic.[1, 2] Rammelkamp and Bradley,[3] on the basis of data showing that diodrast blocked the renal excretion of penicillin, suggested that penicillin was probably excreted by a tubular mechanism. Studies by Rantz and Kirby[4] demonstrated that in afebrile patients 750 to 1,120 ml. of plasma could be cleared of penicillin per minute. This was proof of the tubular excretion of penicillin since glomerular filtration could account for only a small fraction of such a large rate of excretion.

The possibility of prolonging the blood levels of penicillin by blocking the excretory channel has received considerable attention. Following the observation of Rammelkamp and Bradley referred to in the preceding paragraph, Beyer and associates[5-7] succeeded in decreasing the rate of excretion of penicillin by the use of para-aminohippuric acid. Several reports have appeared in which para-aminohippuric acid has been used with apparent success in clinical penicillin therapy.[7-10] Unfortunately, large amounts of diodrast or para-aminohippuric acid must be given to slow the excretion of penicillin significantly since these agents themselves are rapidly excreted by the renal tubules. The fact that both diodrast and para-aminohippuric acid must be administered intravenously is also a serious handicap to their general use.

Bronfenbrenner and Favour[11] found that the administration of 2.5 Gm. of benzoic acid by mouth every four hours to patients on an unrestricted diet may double the penicillin levels, and that the administration of this amount of benzoic acid to patients in whom fluid and salt intake is restricted may result in an even greater increase in the penicillin blood concentrations.

Recently Beyer[12] reported that the excretion of penicillin could be reversibly inhibited by 4′-carboxyphenylmethanesulfonanilide, a compound synthesized by Sprague and co-workers and called caronamide.[13] According to Beyer and co-workers,[14] this compound, while capable of blocking the tubular excretion of penicillin, is not itself subject to a significant degree of tubular excretion. Toxicity studies[15] showed caronamide to have an acute oral LD_{50}

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of 1,405 mg. per kilogram for mice; 1,320 mg. per kilogram for rabbits; and 1,575 mg. per kilogram for dogs. No evidence of histologic damage or hematologic disturbances was observed in rats given 0.5 Gm. per kilogram of caronamide daily for four weeks or in dogs receiving daily doses as high as 1.5 Gm. per kilogram for the same length of time. Verwey and Miller[16] found that caronamide administered orally increased the effectiveness of a given intramuscular dose of penicillin against experimental infections in mice. They believe that the enhancement of penicillin activity by caronamide is due to the effect of the agent on the penicillin concentrations in the body rather than to any synergistic action.

Rapoport and associates[17] studied the toxicity of caronamide and its effectiveness in inhibiting penicillin excretion in fifteen children. In none of their patients did the drug have to be withdrawn because of toxicity when administered in oral doses of 0.1 to 0.4 Gm. per kilogram per day for one or two weeks. Three of their patients developed a rash. They found that 0.2 Gm. per kilogram per day of caronamide given orally increased the plasma levels of penicillin significantly, and that 0.4 Gm. per kilogram per day resulted in a marked rise in penicillin levels. No irreversible changes in glomerular filtration, renal plasma flow, maximum tubular excretory capacity (P. A. H. Tm), or maximum reabsorptive capacity (glucose Tm) were found.

Crosson and co-workers[18] in preliminary studies in adults found that 2 Gm. of caronamide given every four hours enhanced penicillin blood levels when the penicillin was given orally in a dose of 100,000 units or intramuscularly in a dose of 50,000 units. They found that administration of 1 Gm. of caronamide every two hours in similar subjects had little effect on the penicillin levels, while 1.5 Gm. every three hours definitely increased the penicillin levels. It is of interest that in the only patient to whom they gave 100,000 units of penicillin intramuscularly every four hours they failed to obtain increased penicillin levels with 2 Gm. of caronamide every four hours.

These findings seemed sufficiently encouraging to justify further study of caronamide, particularly in relation to the use of large doses of penicillin, with a view to the possibility of decreasing the frequency of injections while maintaining significant concentrations in the blood.

MATERIALS AND METHODS

Choice of Subjects.—The subjects were male hospital patients who were given penicillin, some of them during an acute illness but most of them during convalescence from various infections. Since it is known that advanced renal disease may interfere with penicillin excretion,[2] no patient was chosen in whom there was impairment of renal function as judged by the inability to concentrate urine or to maintain a normal concentration of nonprotein nitrogen in the blood. Otherwise the subjects were selected without regard to their disease. The patients were given the usual hospital diet and no restrictions were placed on their fluid or salt intake. Urine analyses and blood nonprotein nitrogen determinations were done before and during the caronamide administration.

Penicillin and Caronamide Administration.—All of the penicillin doses were given intramuscularly at eight-hour intervals. Crystalline sodium penicillin G, as available commercially, was used throughout and was given as a solution in normal saline in a concentration of 100,000 units per milliliter. The caronamide was supplied in 0.5 Gm. tablets and was given in various doses by mouth every four hours so that every other dose was given at the time of an intramuscular penicillin injection.

In the studies on the effect of caronamide on penicillin levels, the subjects were kept on a constant dosage of 100,000 units of penicillin every eight hours throughout the time when the caronamide was given, and for at least two days before the first dose, and for two, or more often three, days after the last dose of caronamide. The caronamide was usually given for twenty-four hours, and in a few instances during a forty-eight hour period, in order to permit the drug to reach an equilibrium in the body fluids.

Penicillin Blood Levels.—Blood for penicillin levels was obtained during the eight-hour intervals after the last dose of penicillin given during the period of caronamide administration. Two control series of blood were also obtained during similar eight-hour periods, one series just prior to the first dose, and the other two or three days after the last dose of caronamide. The blood was obtained just before and one-half, four, six, and eight hours after the penicillin injection. The first blood in each series was obtained, in each instance, at about 9 A.M. The concentrations of penicillin were determined on citrated plasma by the serial dilution method of Rammelkamp,[19] modified only by the use of 0.5 ml. amounts of the plasma dilutions.*

RESULTS

Effect of 2 Gm. of Caronamide Every Four Hours.—The plasma penicillin levels obtained in twelve subjects before, during, and after a period when caronamide was given in doses of 2 Gm. every four hours are shown in Table I. One-half of the subjects were under 60 years of age; in none of them was there any significant difference in the levels obtained while they received caronamide as compared with the control periods before and after the caronamide administration.

A marked effect from the caronamide was obtained in five of the six subjects over 60 years of age. The effect varied considerably in the different individuals. The levels obtained one-half hour after the dose of penicillin, which probably represent the maximum attained in most patients, were essentially the same (considering the limitations of the method) during the control periods and during the caronamide administration. At four and six hours after the penicillin injections, the levels obtained during the caronamide ingestion ranged from four to sixteen times those found during the control periods. At the beginning and end of the eight-hour interval, the levels in the

*The end point was taken as the dilution in which there was no visible growth of the test organism in broth and on subculture to blood agar. In the occasional serum in which there was no visible growth in the broth and one or more colonies of streptococci developed on the blood agar, the level was taken as the mean of that concentration and the one in which no growth occurred on subculture.

TABLE I. PLASMA PENICILLIN LEVELS DURING INTRAMUSCULAR ADMINISTRATION OF 100,000 UNITS OF PENICILLIN EVERY EIGHT HOURS WITH AND WITHOUT ORAL CARONAMIDE, 2 GM. EVERY FOUR HOURS

SUBJECTS UNDER 60 YEARS OF AGE								SUBJECTS OVER 60 YEARS OF AGE							
CASE	AGE	CARONAMIDE (GM. EVERY 4 HR.)	HOURS AFTER PENICILLIN DOSE					CASE	AGE	CARONAMIDE (GM. EVERY 4 HR.)	HOURS AFTER PENICILLIN DOSE				
			0	½	4	6	8				0	½	4	6	8
1	58	0	0*	2.0	0.03	0	0	7	70	0	0	1.5	0.03	0.01	0
		2	0	1.0	0.03	0	0			2	0.12	2.0	0.5	0.25	0.12
		0	0	2.0	0.02	0	0			0	0	4.0	0.05	0	0
2	36	0	0	0.5	0	0	0	8	86	0	0.03	4.0	0.12	0.05	0.02
		2	0	0.5	0.02	0	0			2	0.25	3.0	1.0	0.75	0.5
		0	0	0.25	0.02	0	0			0	0.02	1.0	0.12	0.03	0.03
3	44	0	0	0.5	0.02	0	0	9	69	0	0.02	1.0	0.03	0.02	0
		2	0	1.0	0.02	0	0			2	0.03	1.0	0.12	0.06	0.03
		0	0	0.25	0	0	0			0	0	0.25	0.02	0.02	0.02
4	46	0	0	1.0	0.02	0	0	10	63	0	0	1.0	0.09	0.06	--
		2	0	1.0	0.03	0	0			2	0.25	1.0	0.5	0.5	0.25
		0	0	0.5	0	0	0			0	--	--	--	--	--
5	46	0	0	2.0	0	0	0	11	73	0	0.06	2.0	0.18	0.12	--
		2	0	1.0	0.02	0	0			2	0.5	4.0	1.0	1.0	0.5
		0	0	1.0	0	0	0			0	0.03	1.0	0.12	0.06	0.03
6	50	0	0	1.0	0.03	0.02	0	12	65	0	0	2.0	0.06	0.02	--
		2	0.02	1.0	0.06	0.02	0			2	0.02	1.0	0.06	0.02	0
		0	0	1.0	0.03	0	0			0	0	1.0	0.03	0.02	0
Average		0	0	1.17	0.02	0	0	Average		0	0.02	1.92	0.09	0.05	0
		2	0	0.92	0.03	0	0			2	0.20	2.0	0.53	0.43	0.23
		0	0	0.83	0.01	0	0			0	0.01	1.45	0.07	0.02	0.02

--, Not done.
*All values <0.01 unit are listed as 0.

same five subjects ranged from 0.03 to 0.5 unit per milliliter of plasma during the caronamide administration and from < 0.01 to 0.06 unit during the control periods. The average increase in the levels for all the six subjects over 60 years of age was about six- and ninefold at four and six hours, respectively. The average level at the beginning and end of the eight-hour interval during caronamide administration was 0.20 and 0.23 unit per milliliter, respectively, as compared with 0.02 unit or less at the same times during the control periods.

Effect of 3 Gm. of Caronamide Every Four Hours in Persons Under 60 Years of Age.—The plasma penicillin levels obtained in four subjects under 60 years of age (Cases 13 to 16) who were given 3 Gm. of caronamide every four hours are shown in the upper part of Table II. In none of these four subjects was there any appreciable effect from the caronamide, the levels throughout the eight-hour period being essentially the same while the drug was given and during the control periods.

Effect of 4 Gm. of Caronamide Every Four Hours in Subjects Under 60 Years of Age.—Ten patients under 60 years of age (Cases 17 to 26) were given 4 Gm. of caronamide every four hours. The penicillin levels resulting from an intramuscular dose of 100,000 units of penicillin in these subjects before, during, and after the caronamide administration are also shown in Table II. An appreciable effect was obtained from the caronamide in every one of these ten subjects, but the effect varied widely in the different individuals. The levels obtained at one-half hour were higher during the caronamide administration in eight of the ten subjects. At four hours the levels during the caronamide period were from four to thirty-two times higher than at the same time during the control periods. At six hours only one subject failed to show an increased level; in the other nine the concentrations of penicillin were two to sixteen times higher during the caronamide administration. Demonstrable levels were obtained in most of the subjects at the end of eight hours (that is, just before the penicillin dose) while they were receiving caronamide but not during the control periods.

For the ten subjects under 60 years of age, the average plasma penicillin level eight hours after an intramuscular dose of 100,000 units of penicillin (or just before such a dose) during the oral administration of 4 Gm. of caronamide every four hours was 0.03 unit per milliliter. At six hours the average level was 0.08 unit per milliliter. Practically none was demonstrable at these times during the control periods in the same subjects. The average levels of penicillin one-half hour and four hours after the penicillin injections were, respectively, about two and ten times higher during the caronamide administration than they were during the control periods.

Effect of Increasing the Dose of Penicillin.—An attempt was made to estimate roughly the intramuscular dose of penicillin which, given at eight-hour intervals, would be required to maintain blood concentrations (particularly during the latter half of that interval) approximating those that resulted from injections of 100,000 units every eight hours during the oral administration of the minimum effective amounts of caronamide. For that purpose another group of subjects were given intramuscular doses of 100,000 units every eight

TABLE II. PLASMA PENICILLIN LEVELS DURING INTRAMUSCULAR ADMINISTRATION OF 100,000 UNITS OF PENICILLIN EVERY EIGHT HOURS WITH AND WITHOUT CARONAMIDE, 3 OR 4 GM. EVERY FOUR HOURS (SUBJECT UNDER 60 YEARS OF AGE)

CASE	AGE	CARONAMIDE (GM. EVERY 4 HR.)	HOURS AFTER DOSE OF PENICILLIN				
			0	½	4	6	8
13	51	0	0*	1.0	0.02	0.02	0
		3	0	0.75	0.03	0.03	0.02
		0	0	1.0	0.03	0	0
14	42	0	–	--	--	--	--
		3	0	0.75	0.02	0	0
		0	0	1.0	0.02	0	0
15	31	0	0	0.5	0.02	0	0
		3	0	1.0	0.03	0	0
		0	0	0.5	0	0	0
16	14	0	0	1.5	0.02	0	0
		3	0	1.0	0.02	0	0
		0	0	1.0	0.02	0	0
Average		0	0	1.0	0.02	0.01	0
		3	0	0.88	0.03	0.01	0
		0	0	0.88	0.02	0	0
17	39	0	0	1.0	0.01	0	0
		4	0.03	2.0	0.12	0.03	0
		0	0	0.5	0	0	0
18	21	0	0	1.0	0	0	0
		4	0.03	2.0	0.12	0.03	0.02
		0	0	0.5	0	0	0
19	16	0	0	1.0	0.01	0	0
		4	0.06	2.0	0.25	0.06	0.03
		0	0	1.0	0.02	0	0
20	27	0	0	2.0	0.03	0.02	0
		4	0.02	2.0	0.25	0.12	0.03
		0	0	1.5	0.03	0.02	0
21	48	0	0	1.0	0.03	0	0
		4	0	2.0	0.12	0.06	0.02
		0	0	1.5	0.03	0.02	0
22	24	0	0	0.75	0.02	0.02	0
		4	0.03	4.0	0.06	0.02	0
		0	0	0.5	0.02	0	0
23	36	0	0	1.0	0.02	0	0
		4	0.06	1.0	0.25	0.12	0.06
		0	0	0.75	0.02	0	0
24	53	0	0	1.0	0.02	0	0
		4	0.02	2.0	0.06	0.02	0
		0	0	0.5	0.02	0	0
25	52	0	--	--	--	--	--
		4	0.03	1.0	0.25	0.12	0.06
		0	0	0.5	0.02	0	0
26	42	0	0.02	0.5	0.02	0	0
		4	0.06	4.0	0.5	0.25	0.06
		0	0	2.0	0.02	0	0
Average		0	0	1.03	0.02	0	0
		4	0.03	2.20	0.20	0.08	0.03
		0	0	0.93	0.02	0	0

--, Not done.
* All values <0.01 unit are listed as 0.

hours for one or two days. This was followed by 200,000 units every eight hours and finally 300,000 units every eight hours for similar periods. A series of blood penicillin levels were obtained during an eight-hour interval on each dose, just as in the previous studies.

Since it was shown that the blood levels on doses of 100,000 units were much better sustained in patients over 60 years of age than in those under

60 years, subjects of both age groups were chosen. The plasma penicillin levels obtained on these three dose levels in six subjects under 60 years and in a similar number of patients over 60 years of age are shown in Table III.

The expected increases in the penicillin levels with the progressive increases in the dose were noted, but there were considerable individual variations. Although marked differences were noted between the height of the levels obtained with any given dose in the two age groups, the increases in levels resulting from increasing the size of the dose were quite similar in both age groups.

Comparison of Effects Obtained From Caronamide With Those Obtained From Increasing the Dose of Penicillin.—In order to compare the effect of caronamide with that obtainable by increasing the dose of penicillin, it would be desirable to carry out all of the studies in the same subjects. This, however, was not feasible. A rough estimate of the comparative effects may nevertheless be made from the data already presented. For that purpose it is best to consider the subjects in the two age groups (that is, those under 60 years of age and those over 60 years of age) separately.

The comparison in the subjects over 60 years of age may be made from the data shown for Cases 7 to 12 in Table I and for Cases 33 to 38 in Table III, particularly from the average values given in these tables. A similar comparison in the subjects under 60 years of age may be made from the levels shown in Table II (Cases 17 to 26) for the effects of 4 Gm. of caronamide and those given for Cases 27 to 32 in Table III. The average values for these groups are shown graphically in Fig. 1. In this figure the averages for the control levels obtained before and after caronamide are included in a single curve.

The curves representing the average levels following increasing doses of penicillin were obtained in the same patients. Those representing the effect of caronamide, however, are averages from other groups of patients. It should be noted that all of the latter patients had been on caronamide therapy for at least sixteen hours before the penicillin levels were determined so that there were appreciable penicillin serum concentrations at zero hour on these curves.

The difference in the declining slope of the curves between the patients under and those over 60 years of age is striking. In the older patients a significant concentration of penicillin persisted in the serum eight hours after the injection of 100,000 units, even when caronamide was not given, whereas in the younger subjects little or no penicillin was detectable six hours after such an injection. Increasing the dose of penicillin altered the height of the curves to a similar extent in both age groups but did not significantly alter the slope of these curves. The caronamide, on the other hand, increased the peak penicillin levels to some extent, but its effect on slowing the rate of fall of the plasma penicillin concentrations was striking. Except at the time of the peaks, the patients receiving the caronamide and 100,000 units of penicillin had significantly higher levels than those receiving 300,000 units of penicillin without caronamide. This difference was greatest in both age groups six

TABLE III. PLASMA PENICILLIN LEVELS DURING INTRAMUSCULAR ADMINISTRATION OF INCREASING AMOUNTS OF PENICILLIN GIVEN EVERY EIGHT HOURS WITHOUT CARONAMIDE

SUBJECTS UNDER 60 YEARS OF AGE								SUBJECTS OVER 60 YEARS OF AGE							
CASE	AGE	PENICILLIN (UNITS EVERY 8 HR.)	HOURS AFTER PENICILLIN DOSE 0	½	4	6	8	CASE	AGE	PENICILLIN (UNITS EVERY 8 HR.)	HOURS AFTER PENICILLIN DOSE 0	½	4	6	8
27	36	100,000	0*	1.0	0.02	0	0	33	60	100,000	0.01	2.0	0.06	0	0
		200,000	0	2.0	0.03	0	0			200,000	0	4.0	0.12	0.03	0.02
		300,000	0	4.0	0.06	0.02	0.02			300,000	0.02	4.0	0.25	0.03	0.02
28	39	100,000	0	0.5	0.02	0	0	34	77	100,000	0.02	1.0	0.12	0.02	0.02
		200,000	0	2.0	0.06	0.02	0			200,000	0	4.0	0.25	0.12	0.03
		300,000	0.02	--	0.12	0.03	0.03			300,000	0.12	8.0	0.5	0.12	0.06
29	57	100,000	0	2.0	0.03	0.02	0	35	73	100,000	0.12	2.0	0.5	0.12	0.12
		200,000	0	2.0	0.25	0.03	0.02			200,000	0.25	2.0	0.8	0.5	0.25
		300,000	0.02	6.0	0.25	0.06	0.06			300,000	0.25	4.0	1.0	0.5	0.25
30	52	100,000	0	0.75	0.03	0	0	36	79	100,000	0.03	2.0	0.12	0.03	0.02
		200,000	0	2.0	0.09	0.02	0			200,000	0.03	2.0	0.18	0.06	0.03
		300,000	0	8.0	0.12	0.03	0.03			300,000	--	--	--	--	--
31	14	100,000	0	2.0	0.02	0	0	37	74	100,000	0	1.0	0.06	0	0
		200,000	0.02	2.0	0.02	0	0			200,000	0	2.0	0.06	0.02	0
		300,000	0	8.0	0.03	0	0			300,000	0.02	4.0	0.5	0.06	0.02
32	17	100,000	0	2.0	0.05	0.02	0	38	78	100,000	0	4.0	0.25	0.03	0.02
		200,000	-	4.0	0.12	0.03	0.02			200,000	0.03	2.0	0.25	0.06	0.02
		300,000	0.02	8.0	0.25	0.03	0.02			300,000	0.06	4.0	0.5	0.25	0.12
Average		100,000	0	1.38	0.03	0.01	0	Average		100,000	0.03	2.0	0.19	0.03	0.03
		200,000	0	2.33	0.10	0.02	0.01			200,000	0.05	2.67	0.28	0.13	0.06
		300,000	0.01	6.80	0.14	0.03	0.03			300,000	0.09	4.8	0.55	0.19	0.09

--, Not done.

*All values <0.01 unit are listed as 0.

hours after the penicillin injection. The levels obtained with 100,000 units of penicillin (without caronamide) were comparable within each age group in both series of studies.

Toxicity.—There was no clinical or laboratory evidence of toxicity from the caronamide in any of the subjects. In no instance was crystalluria, hematuria, or albuminuria observed which could be attributed to caronamide. No

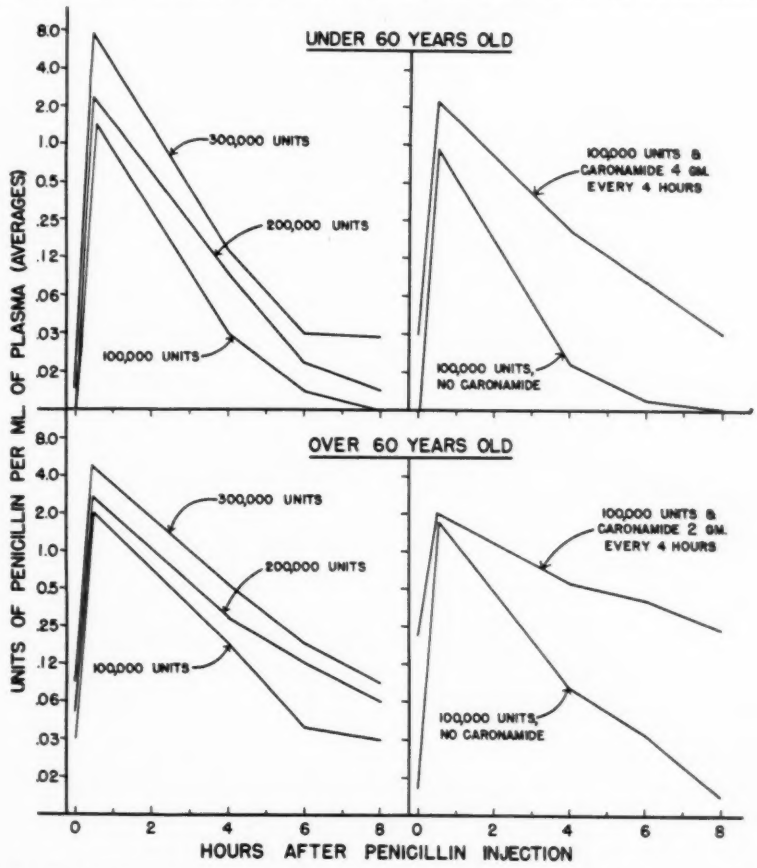

Fig. 1.—Enhancement of plasma levels by caronamide during intramuscular administration of 100,000 units of penicillin every eight hours; comparison with effect of increasing the dose in subjects within the same age group.

significant increase in the level of blood nonprotein nitrogen occurred. Furthermore, the reducing substance reported by Beyer and co-workers[15] as occurring in the urine of animals during administration of caronamide in large doses was not encountered in any of these cases. The findings in the present cases, however, must be interpreted in the light of the brief period during which the caronamide was given.

COMMENT

These studies confirm the findings previously mentioned and show conclusively that caronamide administered by mouth in adequate doses signifi-

cantly enhances penicillin blood levels. Crosson and associates[18] reported that 2 Gm. of caronamide did not have an appreciable effect on the penicillin levels in one of their patients who was receiving 100,000 units of penicillin intramuscularly, whereas the dose was effective in other patients receiving 50,000 units of penicillin. While the age of their patient is not given, the data presented in the foregoing show that 4 Gm. of caronamide is necessary to enhance penicillin levels in persons under 60 years of age who are receiving 100,000 units of penicillin intramuscularly every eight hours and have reasonably good renal function.

Rapoport and co-workers[17] noted significant increases in penicillin blood levels in children with the use of 0.2 Gm. of caronamide per kilogram per day. In adults averaging 60 kilograms in weight, the corresponding dose (namely, 12 Gm. per day) was found adequate for the purpose of increasing the blood levels from the intramuscular dose of penicillin used here only in subjects over 60 years of age. In younger adults twice this amount was necessary to obtain a similar result.

The relative effect of any given dose of caronamide on the blood levels obtained from varying doses of penicillin was not studied.

A comparison of the curves in Fig. 1 shows that the rate of disappearance of penicillin from the blood is significantly slower in the patients over 60 years than in the younger subjects. Since penicillin is excreted chiefly by the tubules, even a major diminution in glomerular filtration could not explain the difference in excretion between the younger and older groups of patients. Presumably, therefore, the reduced rate of disappearance of penicillin from the blood of the older patients is due to a reduction in their functional tubular mass.

Examination of Fig. 1 also shows that the administration of caronamide alters the penicillin blood level curve in young people in a manner qualitatively similar to the alteration apparently caused by age alone. Furthermore, the fact that less caronamide is required to enhance the penicillin levels significantly in the older patients is further evidence of reduced tubular excretory function in the older people.

SUMMARY AND CONCLUSIONS

Caronamide administered orally in doses of 2 Gm. every four hours to patients over 60 years of age markedly enhanced the plasma penicillin levels resulting from the intramuscular administration of 100,000 units of penicillin every eight hours. It was necessary to increase the caronamide dosage to 4 Gm. every four hours in order to produce a similar effect in younger persons. The penicillin blood levels following intramuscular administration of 100,000 units of penicillin every eight hours to patients receiving approximately minimal effective oral doses of caronamide were significantly higher than those obtained by giving 300,000 units of penicillin alone to similar subjects.

The authors are indebted to Dr. William P. Boger, Sharp & Dohme, Inc., Philadelphia, Pa., for the supply of caronamide and for sending us the manuscripts dealing with this drug prior to publication.

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ENHANCEMENT OF PLASMA PENICILLIN CONCENTRATIONS BY CARONAMIDE AND SODIUM BENZOATE

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INTRODUCTION

A CONSIDERABLE number of drugs and vehicles have been introduced into clinical practice in an attempt to improve the practical therapeutics of penicillin by enhancing and prolonging its effective concentration in the blood. Among other agents, benzoic acid and sodium benzoate have been used rather widely to elevate and prolong plasma penicillin concentrations. Despite their use, however, relatively little data are available with regard to their efficacy. Bronfenbrenner and Favour[1] found that blood penicillin concentrations were doubled when benzoic acid was also administered. Bohls and coworkers[2, a, b] reported measurable blood concentrations of penicillin for twenty-four or more hours following both intramuscular and oral administration of an aluminum-penicillin mixture with benzoic acid or sodium benzoate. These results were not confirmed by Spaulding and associates,[3] who found only a moderate enhancement of plasma penicillin concentrations, in some instances for six hours, following the oral administration of several preparations of penicillin with sodium benzoate.

Beyer and associates have introduced a new substance, caronamide, which was found to be effective in enhancing and prolonging penicillin plasma concentrations.[4] Caronamide (4′-carboxy-phenylmethanesulfonanilide) is a stable, crystalline, colorless compound with little or no odor or taste, only slightly soluble in water, and relatively stable to hydrolysis with acids or alkali. In renal clearance studies in dogs it was demonstrated that caronamide decreased tubular excretion and increased the plasma concentration of penicillin.[5] When caronamide was given in a dosage of 50 mg. per kilogram the renal clearance of penicillin approximated that of creatinine, indicating an essentially complete suppression of tubular excretion of penicillin. This effect, which was reversible, persisted for about four hours. Further renal clearance studies indicated that caronamide did not influence the Tm of glucose or arginine, or the clearances of urea, sulfonamides, or creatinine.[6] As would be predicted, however, it decreased the clearance of para-aminohippuric acid. The mechanism of action of caronamide appears to stand in sharp contrast to para-aminohippurate and diodrast. The latter drugs depress penicillin excretion by a mass competition or blockade phenomenon.[7, 8] Caronamide, in contrast, is believed to be excreted wholly through the glomerular filtrate. Because caronamide depressed the tubular excretion of penicillin but was not itself excreted

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through the tubules, Beyer postulated that this substance exerts a selective inhibitory effect on the enzymatic mechanism of the renal tubules which effects the elimination of penicillin.[4]

Caronamide was found to exhibit a low order of both acute and chronic toxicity in laboratory animals.[9] When the drug was given to children for periods of one to two weeks, serious toxic reactions were not observed. The oral administration of caronamide and penicillin resulted in an increase in plasma penicillin concentrations of from 1.8- to 14.5-fold over the concentrations obtained during control periods.[10] Similar results with caronamide were also observed in adults who received penicillin intramuscularly and orally.[11]

These results appeared to be of sufficient interest to warrant the employment of caronamide under clinical conditions. Approximately thirty-five subjects received caronamide by mouth together with penicillin (1) by mouth, (2) by intramuscular injection in aqueous solution, and (3) by intramuscular injection in peanut oil and beeswax. The effect of caronamide on the plasma concentration of streptomycin was also studied in four individuals.

When it became apparent that caronamide did have an effect on the plasma penicillin concentration, the study was extended to include a comparison of the effectiveness of caronamide and sodium benzoate, individually and in combination, in eight additional subjects.

MATERIALS AND METHODS

The subjects used were all young adults and were either healthy physicians, medical students, and nurses or patients convalescent from a variety of illnesses. The twelve subjects who received penicillin in peanut oil and beeswax had primary or secondary syphilis. None of the subjects had clinical or laboratory evidence of renal disease. All ate a general diet and most of them were ambulatory.

Caronamide* was supplied in tablet form and taken by mouth with water. Individual doses varied from 1½ to 3 Gm. at intervals of two to three hours, as indicated subsequently. Sodium benzoate was put up in soft gelatin capsules and taken by mouth with water, in 2 Gm. doses. The sodium salt of amorphous penicillin was used for intramuscular injection. Penicillin in peanut oil and beeswax was supplied as the calcium salt of amorphous penicillin and contained 300,000 units per milliliter. The preparation of penicillin for oral administration was in the form of tablets, each containing 50,000 units of potassium penicillin G with aluminum hydroxide.*

In all of the studies, each subject served as his own control. On the first day of the test penicillin was given alone, at the intervals and in the dosages indicated subsequently. On the following day, the same dosage schedule of penicillin was resumed and, in addition, either caronamide or sodium benozate, or both, were administered. Oxalated blood samples were obtained at the same hours on each day of the study.

An attempt was made to administer the drugs between meals, allowing an hour between medication and the ingestion of food. Subjects were asked to

*Supplied by Dr. J. W. Crosson, of Sharp & Dohme, Inc., Philadelphia, Pa.

maintain the same diet and fluid intake on each day of the test, but no restrictions were imposed on salt or water intake. Close control of the schedules of meals and medication were not always possible, and it is not known, in some instances, how carefully the instructions were followed.

Plasma penicillin concentrations were determined by a modification of the serial dilution method of Rammelkamp.[12] The medium used was brain-heart infusion broth.* The test organism was a hemolytic streptococcus (strain No. 98†) which was inhibited by 0.008 unit of penicillin per milliliter of broth. The end points of the plasma specimens were determined by the presence or absence of growth of explants on blood agar from the tubes with the highest dilutions of plasma which were clear after twenty-four hours' incubation. Despite the recognized inaccuracies of the method, replicate determinations on the same samples yielded the same results. The method is believed accurate for the detection of concentrations of 0.03 unit or more of penicillin per milliliter of plasma.

Caronamide and sodium benzoate in concentrations of 1 mg. per milliliter of broth did not inhibit the growth of the test organism. These concentrations of the drugs, alone and in combination, did not interfere with the in vitro activity of penicillin, in either broth or diluted plasma. Moreover, the plasma obtained from an individual one and one-half hours after the ingestion of 3.0 Gm. of caronamide did not affect the activity of penicillin in vitro. It was concluded, therefore, that the effects of these agents on plasma penicillin concentrations were not due to interference with the sensitivity of the penicillin assay procedure itself.

RESULTS

Effect of Caronamide.—

Penicillin Administered by Mouth: The effect of caronamide on orally administered penicillin was studied in seventeen tests performed on thirteen subjects. In twelve tests (ten subjects) 100,000 units of penicillin were taken by mouth at two-hour intervals from 8 A.M. to 4 P.M. On the next day, the same dosage of penicillin was resumed and, in addition, the subjects received 2 Gm. of caronamide by mouth every two hours from 6 A.M. to 4 P.M. In six tests blood samples were obtained one hour after each dose of penicillin on both days of the tests; in the other six tests the blood samples were obtained two hours after each dose of penicillin and immediately preceding the next dose. The resulting plasma penicillin concentrations are shown in Tables I and II. There can be little doubt that when these subjects received caronamide, the concentrations of penicillin in the plasma were increased as compared with the control periods. In twenty-five paired tests with blood obtained one hour after each dose of penicillin, the plasma concentrations of penicillin were the same with and without caronamide in three, lower with caronamide only once, and higher with caronamide in twenty-one tests. The increase in penicillin concentration associated with the administration of caronamide was twofold in nine, fourfold in five, and eightfold or more in seven

*Difco Laboratories, Inc., Detroit, Mich.
†Obtained from Dr. M. Finland.

TABLE I. EFFECT OF CARONAMIDE ON PLASMA PENICILLIN CONCENTRATION
(PENICILLIN: 100,000 UNITS, BY MOUTH, EVERY TWO HOURS;
BLOOD SAMPLES, ONE HOUR AFTER EACH DOSE)

SUBJECT	SEX	DRUG	PLASMA PENICILLIN CONCENTRATION (UNITS/ML.)				
			9 A.M.	11 A.M.	1 P.M.	3 P.M.	5 P.M.
P. R.	M	Penicillin alone	<0.03	<0.03	0.22	0.06	0.03
		+ Caronamide	<0.03	<0.03	0.06	0.11	0.06
P. R.	M	Penicillin alone	---	0.03	0.03	0.03	0.03
		+ Caronamide	---	0.11	0.06	0.06	0.11
P. S.	M	Penicillin alone	0.03	0.06	0.03	0.03	<0.03
		+ Caronamide	0.03	0.11	0.22	0.22	0.45
P. S.	M	Penicillin alone	---	0.03	0.11	0.06	0.03
		+ Caronamide	---	0.06	0.22	0.11	0.45
N. W.	F	Penicillin alone	---	0.11	0.03	0.11	---
		+ Caronamide	---	0.45	0.11	0.22	0.90
N. P.	F	Penicillin alone	---	0.03	0.03	0.11	0.06
		+ Caronamide	---	0.45	0.45	0.45	0.45

Caronamide (2 Gm. P.O.) at 6, 8, and 10 A.M., 12 N., and 2 and 4 P.M.
Penicillin (100,000 units P.O.) at 8 and 10 A.M., 12 N., and 2 and 4 P.M.
--, Blood sample not obtained.

TABLE II. EFFECT OF CARONAMIDE ON PLASMA PENICILLIN CONCENTRATION
(PENICILLIN: 100,000 UNITS, BY MOUTH, EVERY TWO HOURS;
BLOOD SAMPLES, TWO HOURS AFTER EACH DOSE)

SUBJECT	SEX	DRUG	PLASMA PENICILLIN CONCENTRATION (UNITS/ML.)			
			10 A.M.	12 N.	2 P.M.	4 P.M.
J. L.	M	Penicillin alone	0.03	0.06	0.03	0.06
		+ Caronamide	0.03	0.11	0.22	0.22
N. M.	F	Penicillin alone	0.03	0.11	0.03	0.06
		+ Caronamide	0.11	0.22	0.22	0.11
N. N.	F	Penicillin alone	0.06	0.06	0.03	0.06
		+ Caronamide	0.11	0.45	0.22	0.22
E. S.	M	Penicillin alone	<0.03	<0.03	0.11	0.03
		+ Caronamide	<0.03	0.03	0.22	0.06
P. S.	M	Penicillin alone	<0.03	0.06	0.06	0.03
		+ Caronamide	0.03	0.06	0.22	0.06
P. R.	M	Penicillin alone	0.03	0.06	0.03	<0.03
		+ Caronamide	0.06	<0.03	0.03	<0.03

Caronamide (2 Gm. P.O.) at 6, 8, and 10 A.M. 12 N., and 2 P.M.
Penicillin (100,000 units P.O.) at 8 and 10 A.M. 12 N., and 2 P.M.

(Table I). Similar results were obtained with the twenty-four paired blood samples obtained two hours after each dose of penicillin (Table II). The plasma penicillin concentrations were the same with and without caronamide in five instances, lower with caronamide once, and higher with caronamide in eighteen tests. The increase was twofold in ten, fourfold in four, and eightfold or more in four. Tabulation of the results obtained both one and two hours after the administration of penicillin indicates that the concurrent administration of caronamide was associated with an enhancement of the plasma penicillin concentrations in 80 per cent of the paired blood samples; in 40 per cent this increase was fourfold or greater as compared with the controls. In terms of actual plasma penicillin concentrations, 62 per cent of the blood samples obtained while the subjects were receiving caronamide had a concentration of 0.1 unit or more of penicillin per milliliter of plasma. In contrast,

only 14 per cent of the samples obtained during the control period, on penicillin alone, had concentrations of 0.1 unit or more of penicillin per milliliter of plasma.

Five subjects received 2 Gm. of caronamide and 100,000 units of penicillin by mouth every three hours during the day. Blood samples were obtained three hours after each dose and immediately preceding the next dose. In three of these subjects, on another day, penicillin alone was given and bleedings obtained on the same schedule. Plasma concentrations of penicillin were low in all instances. Only two of nine samples taken while the subjects were receiving penicillin alone contained measurable amounts of penicillin (0.03 unit per milliliter). When the subjects received caronamide in addition, four of twelve samples contained 0.03 unit per milliliter of penicillin. The advantage of caronamide appeared to be negligible. It is evident, therefore, that while caronamide in the dosage used served effectively to elevate the plasma penicillin concentration for a period of two hours after the oral administration of 100,000 units of penicillin, the effect was dissipated within three hours.

Penicillin Administered Intramuscularly: Two subjects received intramuscular injections of 25,000 units of penicillin and two received 50,000 units of penicillin at two-hour intervals from 8 A.M. to 4 P.M. On the following day, the same dosage schedule of penicillin was resumed and, in addition, the subjects received 2 Gm. of caronamide by mouth every two hours, from 6 A.M. to 4 P.M. The plasma penicillin concentrations obtained one hour after each injection of penicillin are shown in Table III.

TABLE III. EFFECT OF CARONAMIDE ON PLASMA PENICILLIN CONCENTRATION (BLOOD SAMPLES ONE HOUR AFTER EACH DOSE)

SUBJECT	SEX	DRUG	PLASMA PENICILLIN CONCENTRATION (UNITS/ML.)				
			9 A.M.	11 A.M.	1 P.M.	3 P.M.	5 P.M.
Penicillin: 25,000 Units I.M. Every Two Hours							
S. L.	F	Penicillin alone	0.03	0.11	0.06	0.11	0.11
		+ Caronamide	0.22	0.22	0.45	0.45	0.11
C. S.	M	Penicillin alone	0.11	0.06	0.11	0.06	0.11
		+ Caronamide	0.11	0.11	0.22	0.22	0.22
Penicillin: 50,000 Units I.M. Every Two Hours							
C. Y.	F	Penicillin alone	---	0.22	0.22	0.22	0.11
		+ Caronamide	---	0.22	0.90	0.45	0.45
L. P.	F	Penicillin alone	---	0.45	0.11	0.22	0.11
		+ Caronamide	---	0.22	0.22	0.45	0.22

Caronamide (2 Gm. P.O.) at 6, 8, and 10 A.M. 12 N., 2 and 4 P.M.
Penicillin I.M. at 8 and 10 A.M. 12 N., and 2 and 4 P.M.
---, Blood sample not obtained.

In ten paired determinations in subjects on the 25,000 unit dosage schedule, the plasma penicillin concentrations were higher when caronamide was given in eight instances and the same in two instances. Seven of the ten blood specimens obtained when the subjects received caronamide had plasma concentrations of penicillin of 0.2 unit per milliliter or more. When the same subjects received penicillin alone, none of the ten specimens contained more than 0.1 unit of penicillin per milliliter of plasma.

In the subjects who received 50,000 units of penicillin, plasma penicillin concentrations were again higher (in six of eight paired specimens) when caronamide was administered concurrently. In general, the subjects who received caronamide with 25,000 units of penicillin had about the same plasma penicillin concentrations as those who received twice as much penicillin without caronamide.

Caronamide also effectively elevated the penicillin concentrations in the plasma of three other individuals who received 40,000 units of penicillin intramuscularly and 2 Gm. of caronamide by mouth at three-hour intervals during the day (Table VI). Blood samples were obtained one and one-half hours after each dose. Plasma penicillin concentrations were increased twofold or more in ten of twelve paired blood samples.

One of the subjects who had previously been given 50,000 units of penicillin was given two injections of 100,000 units of penicillin intramuscularly at 8 A.M. and 12 N. The following day, he was given the same amount of penicillin at the same hours, and, in addition, took 2 Gm. of caronamide at 6, 8, and 11 A.M. and 2 P.M. Plasma penicillin concentrations obtained one, three, and four hours after each dose of penicillin were identical on each day.*

TABLE IV. EFFECT OF CARONAMIDE ON PLASMA PENICILLIN CONCENTRATION (SINGLE I.M. INJECTIONS OF PENICILLIN)

DOSE OF PENICILLIN	SUBJECT	SEX	WITHOUT CARONAMIDE				WITH CARONAMIDE			
			PLASMA PENICILLIN CONCENTRATION (UNITS/ML.) HOURS AFTER PENICILLIN							
			1	2	3	4	1	2	3	4
25,000 units I.M.	L. W.	F	0.06	<0.03	<0.03	<0.03	0.06	0.03	0.03	<0.03
	E. A.	M	0.06	<0.03	<0.03	<0.03	0.11	0.03	<0.03	<0.03
	G. W.	M	0.03	0.03	0.03	<0.03	0.11	0.06	0.03	<0.03
50,000 units I.M.	H. R.	M	0.22	0.06	0.03	<0.03	0.11	0.06	0.03	0.03
	N. W.	F	0.11	0.03	<0.03	<0.03	0.22	0.06	0.03	0.03
	S. W.	F	0.22	0.06	<0.03	<0.03	0.22	0.11	0.06	0.03
100,000 units I.M.	H. R.	M	0.45	0.06	0.03	<0.03	0.22	0.06	0.06	<0.03
	B. H.	M	0.45	0.06	0.03	<0.03	0.45	0.22	0.11	0.06

Caronamide (2 or 3 Gm. P.O.) one hour before and at the time of administration of penicillin.

In eight subjects, plasma penicillin concentrations were determined at intervals following single intramuscular injections of penicillin. On the afternoon of the same day or the next day, the penicillin injections were repeated and, in addition, 2 or 3 Gm. of caronamide were given one hour before the penicillin injection and again at the time of the injection. The results which are recorded in Table IV indicate no significant effects of caronamide on the plasma penicillin concentrations. The discrepancy between these findings and those recorded in the foregoing may be due to the pharmacologic properties of caronamide. It would appear that the substance is either slowly absorbed or else requires a considerable period of time before it exerts its depressant effects on the renal excretory apparatus.

Penicillin Administered in Peanut Oil and Beeswax: The effect of caronamide on plasma penicillin concentrations eighteen and twenty-four hours after

*Subsequent experience with other subjects has indicated that caronimide is effective in elevating plasma penicillin concentrations as much as fourfold, when doses of 200,000 units of penicillin are administered every three hours.

the injection of 600,000 units of penicillin in peanut oil and beeswax was determined in twelve patients with early syphilis.* On the first day of the test, 600,000 units of penicillin (2 ml.) were administered intramuscularly in the buttock at 2 P.M.; at the same time, 1½ Gm. of caronamide were given by mouth and its administration continued thereafter every two hours until noon of the following day. Blood samples were obtained at 8 A.M. (eighteen hours) and at 2 P.M. (twenty-four hours) on the second day. Immediately after the second blood sample was obtained another injection of 600,000 units of penicillin in peanut oil and beeswax was given in the other buttock. No further

TABLE V. EFFECT OF CARONAMIDE ON PLASMA PENICILLIN CONCENTRATION (PENICILLIN IN PEANUT OIL AND BEESWAX: 600,000 UNITS I.M.)

SUBJECT	SEX	HOURS AFTER PENICILLIN	PLASMA PENICILLIN CONCENTRATION (UNITS/ML.)	
			PENICILLIN ALONE	PENICILLIN + CARONAMIDE
C. P.	M	18	0.03	0.22
		24	<0.03	0.45
D. H.	F	18	0.03	0.11
		24	0.06	0.11
A. E.	M	18	0.03	0.11
		24	<0.03	<0.03
C. B.	M	18	<0.03	0.11
		24	<0.03	0.45
E. P.	M	18	0.03	0.06
		24	<0.03	0.03
R. S.	F	18	<0.03	0.06
		24	<0.03	0.11
W. D.	M	18	<0.03	0.06
		24	<0.03	<0.03
S. G.	M	18	0.11	0.11
		24	0.06	0.11
L. M.	F	18	0.11	0.11
		24	<0.03	0.03
O. F.	M	18	0.03	0.03
		24	0.11	<0.03
M. C.	F	18	0.06	0.03
		24	0.03	0.22
E. B.	M	18	0.06	<0.03
		24	0.11	0.03

Caronamide (1½ Gm. P.O.) every two hours for twenty-four hours.

caronamide was given. Blood samples again were taken eighteen and twenty-four hours after the second injection of penicillin. The results are shown in Table V. In the majority of instances plasma penicillin concentrations were higher when caronamide was also given; however, the results were somewhat erratic and difficult to evaluate. Of twenty-four paired blood samples, the penicillin concentrations were higher when caronamide was given in fifteen, the same with and without caronamide in five, and lower with caronamide than without it in four instances. Less than a measurable amount of penicillin (<0.03 units per milliliter) was present in ten samples obtained in the absence of caronamide and in four samples in the presence of caronamide. Moreover,

*These patients were studied by courtesy of Dr. A. G. Schoch, Dr. L. J. Alexander, and Dr. T. L. Shields, whose cooperation and assistance are gratefully acknowledged.

six of the twelve patients had plasma penicillin concentrations of 0.1 unit or more per milliliter while receiving caronamide, as compared with only two who obtained this concentration of penicillin in the plasma when not receiving caronamide.

Effect of Caronamide on Plasma Streptomycin Concentrations.—The possible value of caronamide in enhancing and prolonging streptomycin plasma concentrations was investigated in four subjects. Two subjects were given intramuscular injections of 0.5 Gm. of streptomycin, at 8 A.M. and 4 P.M., and caronamide was given by mouth every two hours from 6 A.M. to 10 P.M. (total dose, 18 Gm.). Blood samples were obtained two, six, and eight hours after each injection of streptomycin. On the following day the same schedule was followed except for the ommission of caronamide. Within the limits of the method, the plasma concentrations of streptomycin were the same whether or not caronamide was given. Two other subjects received single injections of 0.5 Gm. of streptomycin intramuscularly. Blood samples were obtained at intervals for six hours afterward. On the following day, the streptomycin injections were repeated and 2.0 Gm. of caronamide were given by mouth at two-hour intervals for five doses beginning two hours before the streptomycin injection. In one subject, the concentration of streptomycin in the plasma was from one and one-half to two times higher when the caronamide was administered; in the other subject, however, the streptomycin concentrations for six hours after injection were the same with and without the concurrent administration of caronamide.

Effect of Sodium Benzoate Alone and Together With Caronamide.—

Penicillin Administered Intramuscularly: Three individuals received 40,000 units of penicillin intramuscularly every three hours from 8 A.M. to 5 P.M. on each of three consecutive days. On the first day of the test, 2 Gm. of caronamide were taken by mouth at 6, 8, and 11 A.M., and 2 and 5 P.M. On the third day of the test, 2 Gm. of sodium benzoate were taken by mouth at similar hours. Blood samples were obtained one and one-half hours after each injection of penicillin. The plasma penicillin concentrations are recorded in Table VI.

TABLE VI. EFFECT OF CARONAMIDE AND SODIUM BENZOATE ON PLASMA PENICILLIN CONCENTRATION (PENICILLIN: 40,000 UNITS I.M. EVERY THREE HOURS; BLOOD SAMPLES ONE AND ONE-HALF HOURS AFTER EACH DOSE)

SUBJECT	SEX	DRUG	PLASMA PENICILLIN CONCENTRATION (UNITS/ML.)			
			9:30 A.M.	12:30 P.M.	3:30 P.M.	6:30 P.M.
E. C.	F	Penicillin alone	0.11	0.06	0.06	0.03
		+ Caronamide	0.22	0.06	0.11	0.11
		+ Sodium benzoate	0.22	0.11	0.06	0.06
M. H.	F	Penicillin alone	0.11	0.06	0.06	0.06
		+ Caronamide	0.22	0.11	0.22	0.45
		+ Sodium benzoate	0.22	0.45	0.45	0.06
J. D.	F	Penicillin alone	0.22	0.06	0.06	0.06
		+ Caronamide	0.22	0.11	0.22	0.22
		+ Sodium benzoate	0.06	0.11	0.22	0.11

Caronamide and sodium benzoate (2 Gm. P.O.) at 6, 8, and 11 A.M., 2 and 5 P.M.
Penicillin (40,000 units I.M.) at 8 and 11 A.M., and 2 and 5 P.M.

Both caronamide and sodium benzoate effectively increased plasma penicillin concentrations. The two drugs appeared to be equally effective since the penicillin concentrations were the same with caronamide as with sodium benzoate four times, higher with caronamide than with sodium benzoate five times, and lower with caronamide than with sodium benzoate three times.

Penicillin Administered by Mouth: In three subjects who received penicillin by mouth, a comparison was made of the value of caronamide and sodium benzoate individually and together in increasing the penicillin concentration of the plasma. Penicillin, 100,000 units by mouth, was administered every two hours from 8 A.M. to 2 P.M. on each of four consecutive days. On the first day of the test, 2 Gm. of caronamide were also taken by mouth at 6, 8, and 10 A.M., 12 N., and 2 P.M. On the second day, 2 Gm. of sodium benzoate were taken at the same hours in place of the caronamide. On the third day, neither caronamide nor sodium benzoate was administered with the penicillin. On the fourth day both caronamide and sodium benzoate, 2 Gm. of each, were taken at two-hour intervals from 6 A.M. to 2 P.M. Blood samples were secured two hours after each dose of penicillin on each day of the test. The resulting plasma penicillin concentrations are recorded in Table VII (part of the data is also included in Table II).

TABLE VII. EFFECT OF CARONAMIDE AND SODIUM BENZOATE, INDIVIDUALLY AND IN COMBINATION, ON PLASMA PENICILLIN CONCENTRATION
(PENICILLIN: 100,000 UNITS BY MOUTH EVERY TWO HOURS; BLOOD SAMPLES TWO HOURS AFTER EACH DOSE)

SUBJECT	SEX	DRUG	PLASMA PENICILLIN CONCENTRATION (UNITS/ML.)			
			10 A.M.	12 N.	2 P.M.	4 P.M.
E. S.	M	Penicillin alone	<0.03	<0.03	0.11	0.03
		+ Caronamide	<0.03	0.03	0.22	0.06
		+ Sodium benzoate	0.03	0.06	0.06	0.11
		+ Combination	0.06	0.22	0.22	0.22
P. S.	M	Penicillin alone	<0.03	0.06	0.06	0.03
		+ Caronamide	0.03	0.06	0.22	0.06
		+ Sodium benzoate	0.11	0.06	0.11	0.11
		+ Combination	0.06	0.22	0.90	0.45
P. R.	M	Penicillin alone	0.03	0.06	0.03	<0.03
		+ Caronamide	0.06	<0.03	0.03	<0.03
		+ Sodium benzoate	0.06	0.06	0.06	0.03
		+ Combination	0.22	0.06	0.11	0.06

Caronamide and sodium benzoate, each (2 Gm. P.O.) at 6, 8, and 10 A.M., 12 N., and 2 P.M.
Penicillin (100,000 units P.O.) at 8 and 10 A.M., 12 N., and 2 P.M.

In these subjects, caronamide alone was relatively ineffective in raising the penicillin concentration. A twofold or greater increase (as compared with the controls) was recorded in seven of twelve blood samples, but in only one specimen was there a fourfold increase. Sodium benzoate was more effective than caronamide. Three-fourths of the blood samples obtained when sodium benzoate was taken contained more penicillin than the controls; but here, too, in the majority the increase was not greater than twofold. In contrast, the combination of both caronamide and sodium benzoate resulted in a marked enhancement of plasma penicillin concentrations and yielded better results than either drug alone. A fourfold or greater increase in the penicillin con-

centration was obtained in ten of the twelve blood samples, as compared with the controls. Two-thirds of the blood samples obtained on this regimen contained 0.1 unit of penicillin per milliliter of plasma, and none contained less than 0.06 unit.

It was previously demonstrated that measurable amounts of penicillin did not persist in the blood for three hours after the oral administration of 100,000 units of penicillin, taken alone or with caronamide. It was, therefore, of interest to determine the duration of the effect of both caronamide and sodium benzoate. Two subjects took 100,000 units of penicillin and 2 Gm. each of caronamide and sodium benzoate by mouth every three hours during the day. Blood samples were obtained three hours after each dose of penicillin. In five of six blood samples obtained, measurable concentrations of penicillin (0.03 unit or more) were present.

Toxicity of Caronamide and Sodium Benzoate: Approximately fifty adult subjects took caronamide by mouth. The majority received the drug for one day or less; a few took it for as long as three days. The maximum total daily dosage was 18 Gm.; the majority took 10 to 12 Gm. within a twelve-hour period. Gastrointestinal intolerance was the only evidence of toxicity noted. Two subjects complained of nausea and mild diarrhea, the latter persisting for two days. These symptoms began, however, the day after the administration of caronamide and at a time when the subjects were continuing to take penicillin by mouth. The intolerance cannot clearly be ascribed, therefore, to caronamide. Two other subjects complained of nausea and diarrhea for one day coincident with the ingestion of penicillin, caronamide, and sodium benzoate. Again it is impossible to decide which of the three drugs or whether the combination of them was responsible for the gastrointestinal disturbance. Since this reaction occurred in two of the five subjects who received all three drugs at the time time, it is likely that a considerable percentage of individuals placed on this regimen will be unable to tolerate it.

DISCUSSION

The observation that a combination of caronamide and sodium benzoate resulted in higher penicillin blood concentrations than were attained by the use of each drug alone is of particular interest with regard to the mode of action of these agents. Two possible explanations are suggested: (1) that the dose of each drug was inadequate for maximal effect and (2) that caronamide and sodium benzoate act by different mechanisms. Since a rather fixed dosage schedule of caronamide was used in these studies, the validity of the first explanation cannot be established. It may be noted, however, that the total dose of caronamide usually given within four or six hours was close to that required to produce complete suppression of tubular excretion of penicillin in dogs.[5] The assumption has been made that benzoic acid and sodium benzoate act in a manner similar to diodrast[7] and p-aminohippurate,[8] competing with penicillin for a common renal tubular excretory mechanism. The evidence of Beyer bearing on the mode of action of caronamide has been referred to previously.[4] Recently, evidence has been presented that sodium benzoate has an antipeni-

cillinase effect.[13] It has been suggested that this property, rather than a blocking action on the renal tubules, is responsible for the observed action of sodium benzoate. If such were the case, the apparent synergistic effect of caronamide and sodium benzoate could be explained as the summation of two different mechanisms, each of which helps to maintain the penicillin concentration in the blood. In vitro we have noted that caronamide also exhibits an antipenicillinase effect but do not believe that this action offers an explanation for its behavior in the body, especially since normal pooled plasma also exhibits an antipenicillinase effect which is greater than that of caronamide.

The present studies appear to be sufficiently detailed to indicate the probable value of caronamide as an adjunct to penicillin therapy. It is clear that caronamide is effective in elevating the penicillin concentration in the blood; however, for this purpose, its effectiveness appears to be no greater than that exhibited by sodium benzoate. The choice of either drug would seem therefore to depend in part on the respective toxicity of the two agents. Sodium benzoate is regarded as an innocuous substance,[14] its principal disadvantage being its rather frequent nauseant action. Enough experience has not as yet accumulated to assess the incidence of toxic reactions or idiosyncrasies to caronamide. Until such experience is gained under controlled conditions of observation, its routine use in preference to sodium benzoate is not recommended.

When penicillin was given intramuscularly in aqueous solution, the concurrent administration of caronamide or sodium benzoate resulted in a twofold or greater increase in the concentration of penicillin in the plasma in most instances. However, no studies were made as to the total duration of this effect of caronamide. Essentially the same blood concentrations of penicillin were attained with the injection of 25,000 units of penicillin plus caronamide as resulted from the injection of 50,000 units of penicillin without an adjuvant. Such blood concentrations of penicillin are probably entirely adequate for the control of infections due to organisms ordinarily responsive to penicillin therapy, except for those due to unusually resistant strains. If toxicity and intolerance do not prove to be limiting factors in the use of caronamide, this drug plus 25,000 units of penicillin, intramuscularly, every two hours, will probably result in the maintenance of adequate blood penicillin concentrations. Such a regimen, however, while saving of penicillin, does not obviate the need for repeated injections, which is one of the chief disadvantages of penicillin treatment. From a practical viewpoint, therefore, the chief interest in such substances as caronamide and sodium benzoate lies in their possible value in enhancing the effectiveness of penicillin when given by mouth or in single daily injections in peanut oil and beeswax.

Although it is impossible to define an adequate level of penicillin, experience with in vitro tests indicates that penicillin concentrations of 0.03 to 0.06 unit per milliliter are generally adequate for control of infection with the majority of strains of pneumococcus, hemolytic streptococcus of group A, gonococcus, and *Streptococcus viridans*. From two to eight times as much penicillin may be needed for the control of infections due to some strains of these organisms as well as to many strains of staphylococcus and meningococcus.[15] In

the present study, when 100,000 units of penicillin were taken orally every two hours, adequate plasma penicillin concentrations were not achieved in a large proportion of the blood samples. Two-thirds of the blood specimens obtained one hour after this oral dose of penicillin, and one-half of the specimens obtained two hours after this dose, contained 0.03 unit or less per milliliter.

Caronamide was of definite value in enhancing the penicillin concentration in the plasma when penicillin was given by mouth. In 80 per cent of the blood samples obtained one and two hours after the ingestion of 100,000 units of penicillin, the administration of caronamide was associated with a twofold or greater increase in the plasma penicillin concentration; in 40 per cent the increase was fourfold or greater over the corresponding control samples. When the subjects took caronamide, 70 per cent of the blood specimens contained 0.1 unit or more per milliliter one hour after the dose of penicillin, and 54 per cent of the samples still contained 0.1 unit or more per milliliter two hours after the dose of penicillin. Unfortunately, caronamide did not maintain adequate penicillin concentrations for three hours. The effect of sodium benzoate was essentially the same as that of caronamide for two hours after the oral dose of penicillin. The duration of effect of this drug was not measured beyond two hours, but the data strongly suggested that sodium benzoate likewise does not maintain effective plasma penicillin concentrations for three hours after a dose of 100,000 units of penicillin orally. The combination of sodium benzoate and caronamide together with 100,000 units of penicillin orally, every three hours, resulted in optimal plasma concentrations for two hours and adequate concentrations of penicillin for three hours. Although this regimen appears to yield good results, it seems likely that a considerable number of patients will be unable to tolerate it.

While the foregoing data suggest the value of caronamide or sodium benzoate as adjuvants for the successful use of orally administered penicillin, they omit consideration of a serious limitation; namely, the differences in response which individual subjects exhibited. When oral penicillin was given alone, variations in the plasma concentrations attained were evident not only in different subjects, but also in the same subjects from hour to hour. The time of ingestion of meals and the fluid intake have an important influence on blood concentrations of penicillin,[1, 16] and such influences were undoubtedly responsible for some of the variations observed. When caronamide was taken, however, the individual variations in plasma penicillin concentrations were greatly accentuated. For example, Subject P. R. (Tables I, II, and VII) consistently exhibited a poor response to caronamide and to sodium benzoate; Subject N. P. (Table I), on the contrary, had an unusually good effect from the administration of caronamide. Such unpredictable individual variations, while recognized in the pharmacology of all drugs, have been particularly noted with the oral administration of penicillin.[3] They render uncertain any attempt to define minimal effective dosage schedules for penicillin, which can be used routinely without the necessity of measuring the blood concentrations.

In keeping with the findings of others,[17] the results in the present study indicate that only a minority of patients maintain an effective concentration

of penicillin in the blood twenty-four hours after the intramuscular injection of 600,000 units of penicillin in peanut oil and beeswax. Less than 0.03 unit of penicillin per milliliter of plasma was present at twenty-four hours in seven of twelve patients, and only two had concentrations of 0.1 unit or more. It is, therefore, of interest that when caronamide was administered throughout the twenty-four hours following the injection of penicillin in wax and oil, only three of twelve had plasma penicillin concentrations of less than 0.03 unit per milliliter, and six had 0.1 unit or more per milliliter of plasma. The inconsistencies in the results, however, are such that a definite opinion as to the value of caronamide cannot be expressed. Individual variations are undoubtedly of importance when penicillin is administered by this route as well as by mouth, and, in addition, such factors peculiar to this route of administration as the accuracy of measurement of the dosage, variations in the site of deposition of the penicillin in the tissues, and amount of muscular activity, influence the resulting plasma penicillin concentrations.

The foregoing discussion implies a field of usefulness for caronamide and sodium benzoate which practical considerations may negate. Despite their effect, it is believed that these drugs will prove to be of only limited value when penicillin is given by intermittent intramuscular injection. The saving in the cost of penicillin may be more than offset by the inconvenience and potential toxicity of additional medications.* A similar conclusion probably also will soon apply to penicillin in peanut oil and beeswax. Larger doses in more convenient preparations will obviate the need for adjuvants such as these.

In the field of oral penicillin therapy these substances may have greater usefulness. In terms of the plasma penicillin concentrations attained, oral therapy is not only inefficient and costly, but also unreliable. The addition of caronamide or sodium benzoate, while not permitting any reduction in the dosage of oral penicillin, at least appears to assure more satisfactory blood concentrations than can be obtained without them.

SUMMARY AND CONCLUSIONS

1. The effectiveness of caronamide (4′-carboxy-phenylmethanesulfonanilide) in enhancing the plasma penicillin concentration was studied in thirty-five adult subjects. This effect was compared with that of sodium benzoate.

2. The only toxic effects of caronamide encountered were nausea and mild diarrhea in a few individuals.

3. The administration of 100,000 units of penicillin by mouth resulted in low plasma concentrations one and two hours after each dose. The concurrent administration of caronamide resulted in a twofold or greater increase in plasma penicillin concentrations in 80 per cent of the blood samples, and a fourfold or greater increase in 40 per cent of the samples. This enhancement of the plasma penicillin concentration by caronamide was maintained for two hours but not for three hours after each dose of penicillin.

*Experience acquired since the completion of this manuscript indicates that in situations in which more than a million units of penicillin per day are required, the concurrent administration of caronamide may be of considerable value. Increases of twofold or greater in plasma penicillin levels may be obtained, and result in considerable saving in the amounts of penicillin required.

4. In subjects who received 25,000 and 50,000 units of penicillin intramuscularly, the concurrent oral administration of caronamide likewise resulted in two- to eightfold increases in plasma penicillin concentrations in most instances. When penicillin was injected in peanut oil and beeswax the effects of caronamide were variable, but in the majority of instances plasma penicillin concentrations were increased.

5. Caronamide had no effect on the plasma concentration of streptomycin when the latter substance was administered in multiple intramuscular injections.

6. In general, sodium benzoate was as effective as caronamide in increasing plasma penicillin concentrations when penicillin was given by mouth or by intramuscular injection. The ingestion of both sodium benzoate and caronamide by subjects receiving penicillin by mouth resulted in a greater enhancement of plasma penicillin concentrations than was effected by either of these agents alone.

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A WATER-SOLUBLE PREPARATION FOR PROLONGING EFFECTIVE PENICILLIN LEVELS IN BODY FLUIDS

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THE exigencies of routine clinical practice frequently call for the use of a single daily dose injectible preparation of penicillin that can be relied upon to obtain and maintain effective therapeutic levels of the antibiotic in body fluids. The preparation, ideally, must be stable, water soluble, nontoxic, and nonirritant, and it must be capable of easy and safe administration.

To meet these basic criteria, the following formula* has been developed and employed successfully:

Crystalline sodium salt of penicillin G (300,000 Oxford units)	180.0 mg.
Ephedrine sulfate	25.0 mg.
Epinephrine dihydrochloride	1.0 mg.
Eucupine dihydrochloride	1.0 mg.
Gelatin-dextrose mixture (dried weight)	800.0 to 1,200.0 mg.

When sterilized, dehydrated, and bottled, the preparation appears as a homogeneous powder. On the addition of 2 ml. of distilled water, the powder dissolves readily at room temperature and with great facility if the container is agitated or gently heated by immersion in hot tap water. The resultant amber-colored, water-clear solution may then be drawn up into a 5 or 10 ml. syringe through an 18- or 19-gauge needle and injected without undue haste, subcutaneously or intramuscularly, using a 20-gauge needle. A delay in injection of more than thirty minutes at room temperature may solidify the gel which can then again be fluidified by gentle heat as indicated.

RATIONALE

The vehicle, as described, is composed of penicillin, vasoconstrictors, a local anesthetic, and the gelatin-dextrose mixture. The final pH of the dehydrated preparation, as bottled, is 6.0.

Crystalline sodium penicillin G, 300,000 Oxford units, is the conventional dose for repository injection. It is noteworthy that special penicillins, such as the calcium salt, are not essential for this preparation. If it is desirable to augment the standard dosage, it can be done by merely incorporating additional penicillin in the 2 ml. sterile distilled water which is used to reconstitute the preparation.

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The vasoconstrictors are included for additive prolonging effects. Following the injection, because of the vasoconstrictor drugs, the patient may occasionally complain of transitory palpitation and nervousness. No objective vasoconstrictor effects have been observed in any of the subjects.

The eucupine dihydrochloride serves as a local anesthetic. The hydrogen-ion concentration of 6.0 is optimal. With this pH the penicillin remains stable at room temperature for long periods of time. Furthermore, there is no deterioration of the vasoconstrictor drugs nor is there precipitation of the eucupine dihydrochloride.

In the course of our studies with heparin, the Pitkin menstruum was adopted as the vehicle in order to accomplish a slower and more equable absorption of the drug and to supplant the cumbersome technique of intravenous administration.[1, 2] The Pitkin menstruum was devised for regulating and retarding the rate of release of water-soluble drugs injected subcutaneously or intramuscularly. The clinical deportment of heparin in the Pitkin menstruum has been reported on several occasions.[3-5]

The possibility of utilizing the Pitkin menstruum for penicillin was explored with only partially successful results because both the water content and the low pH induced by the acetic acid in the menstruum were deleterious to penicillin.

These shortcomings were overcome by (1) the selection of gelatins which are more compatible with penicillin and more physiologically acceptable by body tissues, (2) elimination of acetic acid, (3) adjustment of pH, and (4) dehydration of the preparation.

The selection of a satisfactory gelatin* is predicated on several factors. While gelatins are nonallergenic and therefore may be employed in preparing vehicles for intramuscular injections, they differ considerably in their chemical and physical properties, depending upon the source material and method of extraction.

The gelatin of choice must have a suitable molecular weight and jelly strength. To be of practical value this gelatin must lend itself readily to dehydration and reconstitution in aqueous solution at room temperature.

TECHNIC OF ADMINISTRATION

The steps in the administration of the preparation have been previously indicated but may be summarized as follows:

1. Warm the bottle by immersion in hot water and/or by agitation.
2. Expose the rubber diaphragm and sterilize with alcohol; inject 2 ml. of sterile distilled water.
3. Reimmerse the bottle in warm water in a basin or under the tap.
4. With an 18- or 19-gauge needle withdraw the solution into a 5 or 10 ml. sterile syringe.
5. Substitute a 20-gauge needle and inject the gel subcutaneously or intramuscularly, first withdrawing the piston slightly to make sure that a vein has not inadvertently been entered.

*We wish to express our appreciation to Dr. Thomas B. Downey, of the Kind-Knox Gelatin Co., Camden, N. J., for his interest and cooperation in this phase of our problem.

RESULTS

The clinical deportment of the various dehydrated penicillin and gelatin-dextrose preparations has been observed in over 300 subjects. The series included normal controls and ambulatory and bed patients with and without infections. These clinical observations were for the purpose of establishing the composition of the optimum preparation, having in mind its anti-infective properties, its effectiveness in prolonging penicillin levels in body fluids, the absence of toxicity, freedom from local reactions, and ease of administration. Stability studies on the preparation were also carried out with bottles that were stored at room temperature for more than a year.

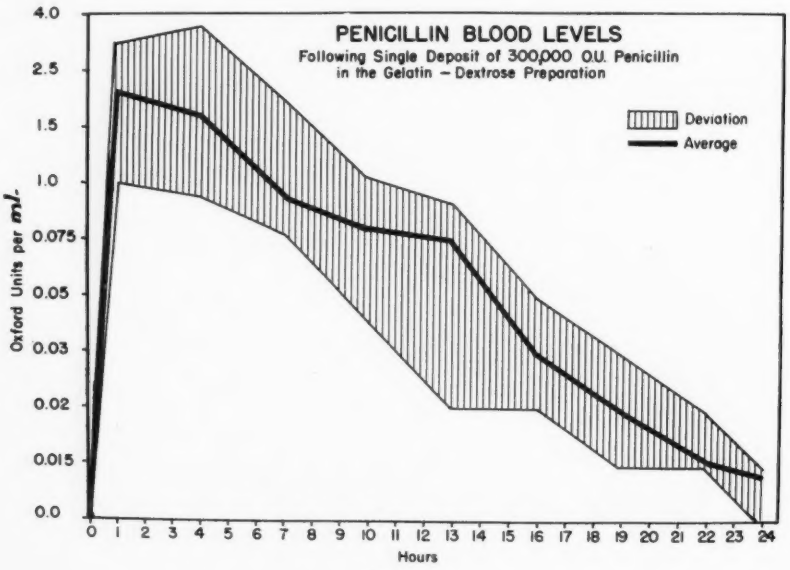

Fig. 1.

Analysis of data (Table I) and a composite graph (Fig. 1) of blood levels* of twenty-four consecutive patients disclose measurable amounts of penicillin up to twenty-four hours following a single dose of 300,000 Oxford units in the gelatin-dextrose preparation. The pattern of blood levels in this group of twenty-four patients is characteristic for all subjects receiving the optimum preparation. The average blood penicillin levels are at peak during the initial eight to thirteen hours (0.08 to 1.91 Oxford units per milliliter) and then taper off to a level (0.015 Oxford units per milliliter) which is still bacteriostatic for many of the ordinary penicillin-inhibitable infecting organisms. These include most strains of gonococci, pneumococci, hemolytic streptococci, and staphylococci. In no instance has the preparation of choice been toxic or productive of local reactions.

*The penicillin assay method used was that described by Rosenblatt, Alture-Werber, Kashdan and Loewe,[6] which employs *Streptococcus pyogenes* C203 as test organism. In a comparative study this system was found by Dolkart, Dey, and Schwemlein[7] to yield generally and, at times, appreciably lower values than the Food and Drug Administration assay procedure described by Randall, Price, and Welch,[8] which uses Bacillus subtilis NRRL B-558 as the test organism.

TABLE I. ANALYSIS OF BLOOD PENICILLIN LEVELS IN A SERIES OF TWENTY-FOUR PATIENTS INJECTED (SUBCUTANEOUSLY OR INTRAMUSCULARLY) WITH 300,000 UNITS OF PENICILLIN IN THE GELATIN-DEXTROSE PREPARATION

NUMBER OF PATIENTS TESTED	1 HR.	4 HR.	7 HR.	10 HR.	13 HR.	16 HR.	19 HR.	22 HR.	24 HR.
	24	24	24	24	24	23	22	16	6
Number of patients with a level of 0.03 units per milliliter or more	24	24	24	24	23	15	4	0	0
Per cent	100	100	100	100	95	65	18	0	0
Average level (from all determinations) units per milliliter	1.91	1.62	0.89	0.35	0.09	0.03	0.03	0	0
Number of patients with a level of 0.015 units per milliliter or more	24	24	24	24	24	23	22	16	3
Per cent	100	100	100	100	100	100	100	100	800'0 50
Average level (from all determinations) units per milliliter	1.91	1.62	0.89	0.35	0.08	0.03	0.02	0.016	

The therapeutic application of this preparation has been studied in various diseases such as gonorrhea, pneumococcus pneumonia, streptococcus sore throat, and furunculosis. The results of these studies will be the subject of subsequent reports.

DISCUSSIONS

Ever since penicillin became available for clinical use, attempts have been made to devise methods for administering this agent which would overcome its inordinately rapid loss through the kidneys. Although this problem has engaged the attention of numerous investigators, recourse, for the most part, must still be made to conventional, fractional intramuscular injections or continuous venoclysis.

Even with two-hour intermittent, intramuscular injections, there is a fraction of the treatment period wherein there is little or no assayable penicillin in the blood. About 60 per cent of the parenterally administered penicillin is excreted in the urine, mostly within one hour after intramuscular injection of the aqueous solution. As a result, in some infections treatment failure is almost inevitable. This is especially true of infections caused by the more resistant types of organisms. Furthermore, in long-continued infections repeated intramuscular injections become irksome and distressing. Continuous intravenous administration is resorted to in these circumstances, usually with gratifying results. The continuous venoclysis route is still the method of choice, however, where uninterrupted massive dosages are required over extended periods of time, as in the treatment of subacute bacterial endocarditis.[9, 10] It may even be necessary to incorporate in the treatment program some potentiating agent such as sodium para-aminohippurate in order to achieve requisite blood penicillin assays.[10-13]

Penicillin has been emulsified in beeswax and peanut oil[14] and in oil and water mixtures[15] as a means of retarding absorption and prolonging the action

of penicillin and thus obviating frequent repeated intramuscular injections necessary with aqueous solutions. While the penicillin assays in body fluids of patients so treated reveal adequate levels for acute infections, the continued use of these foreign insoluble vehicles has certain disadvantages. Local reactions are common,[16] and foreign body tissue responses have been reported following these repositories.[17] Additional drawbacks of the oil and beeswax preparations are lack of stability at room temperature and technical difficulties of administration. For these reasons a water-soluble, wholly absorbable, stable, nontoxic vehicle for penicillin is preferable.

SUMMARY AND CONCLUSIONS

A new penicillin preparation for prolonging effective levels in body fluids has been described. It is water-soluble, nontoxic, free of local reactions, and easy to administer. It maintains measurable levels in the blood for periods up to twenty-four hours or longer and has retained its stability at room temperature for more than a year.

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USE OF MODIFIED DUBOS AND DAVIS MEDIUM FOR DEMONSTRATION OF ANTIBIOTIC ACTIVITY OF SUBTILIN AGAINST MYCOBACTERIUM TUBERCULOSIS

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ATTEMPTS to demonstrate the antibiotic activity of subtilin in vitro against *Mycobacterium tuberculosis* by means of the classical Long's synthetic medium have yielded inconsistent results.[1] With any antibiotic, such as subtilin, which has been shown to lose 68 per cent of its activity when held at 37° C., pH 7.3, for six days,[2] a single, initial application has proved to be inadequate.[3] It is obvious that the results obtained at the end of three weeks' exposure (the usual growth period) do not represent initial activity. In addition, the classical method, which consists of floating the inoculum, does not permit intimate contact between the antibiotic and all mycobacteria present in the culture. In fact, only those organisms present in the base and periphery of the inoculum seem to be affected.

A recently developed liquid medium which appears to circumvent these difficulties is that of Dubos and Davis,[4] who made use of a synthetic wetting against under the trade name of "Tween 80" to facilitate submerged growth, and a purified albumin (Cohn's Fraction V) to increase the growth rate of mycobacteria. In the present communication we wish to report our experience with the use of this medium to demonstrate the antibiotic activity of subtilin against a virulent strain of *Myco. tuberculosis.*

MATERIALS AND METHODS

Myco. tuberculosis† was the organism employed. Two lots of subtilin, one of which was more toxic for mice than the second, were used. The composition of the slightly modified Dubos and Davis medium is given in Table I. The diluted "Tween 80" solution was autoclaved in the same manner as the medium, while the bovine albumin‡ was sterilized by Seitz filtration. All reagents were chemically pure and the pH of the medium was adjusted to 7.2. It was autoclaved at 15 pounds of pressure for fifteen minutes. The medium was incubated for sterility at 37° C. for twenty-four hours before use. All pH determinations were made with the Beckman pH meter. To demonstrate antibiotic activity the scheme of mixing the subtilin solution and the medium, shown in Table II, was adopted. The subtilin solutions were measured by means of

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†Supplied by Dr. Walter Jamieson, Director of the Biological Division of the Lilly Research Laboratories, Indianapolis, Ind.

‡From The Armour Laboratories, Chicago, Ill.

TABLE I

Monopotassium phosphate	1.0 Gm.
Disodium phosphate	6.25 Gm.
Sodium citrate	1.50 Gm.
Magnesium sulfate	0.60 Gm.
Casein hydrolysate, 10% aqueous solution	20.00 ml.
Distilled water to make	1,000.00 ml.
The following were added after sterilization:	
"Tween 80," 10% aqueous solution	5.00 ml.
Albumin, 5% aqueous solution	30.00 ml.

TABLE II

SUBTILIN			
VOLUME IN ML.	DILUTION USED	MEDIUM IN ML.	FINAL CONCENTRATION OF SUBTILIN
0.25	1:1000	4.75	1:20,000
0.125	1:1000	4.88	1:40,000
0.63	1:10,000	4.37	1:80,000
0.50	1:10,000	4.50	1:100,000
0.25	1:10,000	4.75	1:200,000
0.125	1:10,000	4.88	1:400,000
0.63	1:100,000	4.37	1:800,000
0.32	1:100,000	4.78	1:1,600,000
Control		5.00	0

a sterile tuberculin syringe fitted with a 25-gauge needle, while the modified medium was measured with a 5 ml. syringe. All dilutions, including the control, were put into sterile Pyrex test tubes with sterile tightly fitted rubber stoppers to prevent evaporation. Duplicate runs were made by three workers at different times. Inoculation was made from a culture grown in the modified medium for five days, and one loopful of the well-shaken organisms was used for each tube. All tubes were carefully covered with paper and incubated at 37° C. Examination was made each day and every tube was thoroughly shaken before being returned to the incubator. To exclude contamination, cultures were made on blood agar plates, pH 7.6, and smears were stained by a new acid-fast technique[5] and examined microscopically.

For determination of the bacteriostatic or bactericidal property of subtilin, 1.0 ml. of each dilution not showing growth was injected into the inguinal regions of each of two guinea pigs. The inoculum used was twelve days old and was not demonstrably positive at the time of injection. In the case of the control tubes, in which no subtilin was present, growth of *Myco. tuberculosis* was so heavy that 0.2 ml. given per animal was considered adequate for infection. Skin tests on guinea pigs were made intradermally with 0.01 mg. of *P. P. D.* contained in 0.1 ml. of buffered saline, pH 7.0, at the end of the fourth week; readings were made forty-eight hours afterward. All animals were sacrificed at the conclusion of the skin test.*

RESULTS

In the course of this study it was necessary to make a number of preliminary observations on the original Dubos and Davis medium in order to

*The tuberculin was kindly supplied by Dr. Florence Seibert, of the Henry Phipps Institute, Philadelphia, Pa.

determine the optimum conditions required for subtilin activity and for growth of the tubercle bacillus. It was found that the addition of either vegex or glucose, separately or in combination with the medium as originally recommended, did not exert any appreciable effect in increasing the growth rate of our strain. Similarly, glycerol was found to be of little value. A progressively acid pH was obtained when glycerol in the final concentration of 5 per cent was added, whereas a progressively alkaline pH was found when glucose in final concentration of 1 per cent was used. These observations are in agreement with those made by one of us.[6] In both instances the activities of subtilin were distinctly inferior to those obtained with the simplified medium given in this report. In order to initiate rapid growth, it was necessary to use an active growing phase of our strain seeded in Long's synthetic medium employing the filmy portion of growth (between ten and fourteen days). After four to five serial transfers in the Dubos and Davis medium made every fifth day, it was possible to obtain a smooth culture which yielded satisfactory growth at the end of seventy-two hours. Under these conditions it was found that University of California Lots 8 and 10 of subtilin regularly inhibited the growth of the *Myco. tuberculosis* in dilution of 1:400,000.* The rate of growth during a typical experiment is given in Table III.

TABLE III. ANTIBIOTIC ACTIVITY OF SUBTILIN AGAINST MYCOBACTERIUM TUBERCULOSIS IN MODIFIED DUBOS AND DAVIS MEDIUM

DILUTION OF SUBTILIN	DAY				
	3	4	5	7	12
1:20,000	–	–	–	–	–
1:100,000	–	–	–	–	–
1:200,000	–	–	–	–	–
1:400,000	–	–	–	–	–
1:800,000	–	±	+	+	+
1:1,600,000	±	+	++	++	+++
Control	+	++	+++	++++	++++

+, Growth; –, no growth.

Growth at the end of the third day was indicated by the presence of a fine white sediment which, upon microscopic examination, revealed numerous acid-fast bacilli. A turbid supernatant usually indicated contamination. When the tube was vigorously agitated the organisms remained in uniform suspension. On the other hand, no organisms could be demonstrated by the acid-fast technique from the clear tubes; namely, those containing subtilin in concentrations of 1:400,000 or lower. No attempt to concentrate the organisms from these tubes by high speed centrifugation was made since it was thought that positive results would then be attributable to the original inoculum.

The attempt to differentiate bacteriostatic from bactericidal activity in subtilin yielded results which are presented in Table IV.

An examination of Table IV revealed several points of interest. In the first place, control animals were heavily infected when only 0.2 ml. was given to each guinea pig. In the second place, under the conditions of the experiment

*Streptomycin was active at a dilution of 1:800,000 (Y. C. Chin, personal communication).

TABLE IV. RESULTS OF MICROSCOPIC EXAMINATION OF GUINEA PIG TISSUES TO DIFFERENTIATE THE BACTERIOSTATIC AND BACTERICIDAL PROPERTIES OF SUBTILIN

ANIMAL	CONCENTRATION OF SUBTILIN IN INOCULUM	FINDINGS
1 (control)	0	Liver: massive tubercle infiltration Lung: tubercles present throughout
2 (control)*	0	Lymph nodes and spleen: tubercles present Liver: greater involvement than in other tissues
3	1:10,000	No evidence of tuberculosis
4	1:10,000	No evidence of tuberculosis
5	1:20,000	No evidence of tuberculosis
6	1:20,000	No evidence of tuberculosis
7	1:40,000	Lung: numerous early lesions found
8	1:40,000	No evidence of tuberculosis
9	1:80,000	Spleen: tuberculous invasion noted
10	1:80,000	No evidence of tuberculosis
11	1:100,000	Lung: early lesions and areas of consolidation
12	1:100,000	Lung: tubercles present Liver: overwhelming invasion noted Spleen: tubercles present
13	1:200,000	No evidence of tuberculosis
14	1:200,000	Spleen: tubercles present Testis: caseation observed

*Died on the nineteenth day of infection.

1:20,000 dilution or lower of subtilin was definitely bactericidal, while dilutions between 1:40,000 and 1:200,000 of the antibiotic appeared to be bacteriostatic rather than bactericidal. This was substantiated by the finding that five of eight animals inoculated with cultures containing these dilutions revealed various stages of tuberculous infections when animals were sacrificed four weeks after initial infection. In the third place, infections among the experimental animals (Animals 3 to 14) on the whole were less severe than in the controls. This would further indicate that the number of organisms originally present was small since microscopic examinations did not reveal their presence.

A comparison was made between the tuberculin skin test and the macroscopic and the histologic findings in guinea pigs infected with tuberculosis. It was found that histologic studies gave the highest number of positives. Since only one dose of tuberculin was used it is conceivable that animals with early, mild infections may not develop allergy to a degree detectable by 0.01 mg. of P. P. D. In general there is close agreement among the three tests.

COMMENT

The use of modified Dubos and Davis medium has permitted the rapid evaluation of an antibiotic, subtilin, against a virulent strain of *Myco. tuberculosis*, the result of which has, heretofore, been inconclusive. This was effected by incorporating a synthetic wetting agent, "Tween 80," and by the addition of a purified albumin fraction to a simple synthetic medium. The former made possible submerged growth while the latter increased the growth rate of the acid-fast bacilli, all of which favors subtilin standardization.

Although subtilin regularly inhibited the growth of our strain of Mycobacterium in concentration of 1:400,000, subsequent animal inoculation revealed

that its bactericidal property in vitro was low. It required a concentration of 1:20,000 to sterilize the organisms employed in these tests. It may be inferred, therefore, that the chief mode of action of this antibiotic in vivo is bacteriostatic since the concentration of this amount in the blood stream or tissues can hardly be achieved. Unpublished data from this laboratory indicate that subtilin is precipitated in the presence of sodium chloride. Concentrations of subtilin (University of California Lots 8 and 10) greater than 0.1 mg. per cent appear to be precipitated in the blood. Nevertheless, the marked bacteriostatic effect shown here in conjunction with the low toxicity of subtilin are favorable indications for therapeutic trials. A less toxic salt-soluble fraction of subtilin is now available for such trials.[7]

SUMMARY

The modified Dubos and Davis medium is suitable for demonstrating the antibiotic activity of subtilin against a virulent human strain of *Myco. tuberculosis*. Under the conditions of the test a 1:400,000 dilution of subtilin regularly inhibited the growth of these organisms, while dilutions of 1:20,000 or lower were required to achieve bactericidal effect.

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FURTHER OBSERVATIONS ON THE CULTIVATION OF TUBERCLE BACILLI FROM PATHOLOGIC MATERIAL IN DUBOS MEDIA

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INTRODUCTION

THE bacteriologic diagnosis of tuberculosis still is dependent upon the demonstration of the tubercle bacillus by direct microscopic examination, by animal inoculation, by culture, or by various combinations of these methods. Although the guinea pig remains the method of choice, especially with small specimens or specimens of material which are not easily obtainable, the decreasing use of animal injection has placed more and more emphasis on cultures for the diagnosis of those cases which are negative by direct microscopic examination. In the past years several generally satisfactory methods for the cultivation of Mycobacteria have been developed, such as Dorset's,[1] Petragnani's,[2] Löwenstein's,[3] and Schwabacker's[4] variations of coagulated egg media. Most of these, if properly prepared and used with reasonable care, yield a high percentage of positive results. Fundamentally, however, there are two objections which might be made to such media: (1) difficulty in preparation and (2) the time required to obtain growth of tubercle bacilli from pathologic material, often a matter of six or eight weeks.

Dubos[5] recently described a medium which not only supported the growth of minimal inocula of stock strains of tubercle bacilli, but also permitted the rapid growth of cultures planted with such inocula. Foley[6] in a recent study found that such media could be used for the isolation of tubercle bacilli from pathologic material. The purpose of the present study is to report further experience with these media in the bacteriologic diagnosis of tuberculosis.

METHODS

In the previous study[6] several variations of the basic medium as described by Dubos[5] were employed. Since there appeared to be little difference in the results obtained in these various media, further investigation has been limited to the use of one formula, that most recently described by Dubos[7] (Table I).

The mineral base can be prepared as a stock solution and then stored at room temperature with the addition of a few drops of chloroform, the various solutions (and agar if desired) being added just prior to use. Serum albumin must be inactivated before use in media containing "Tween" since it may contain sufficient lipase to split "Tween" into free fatty acids which inhibit the growth of the tubercle bacillus. Dubos[8] recently suggested the addition of sodium fluoride to the medium to inactivate the serum albumin lipase. Preliminary experiments in this laboratory with the H37RV strain indicate that as little as 0.005 per cent sodium fluoride is sufficient to prevent lipase activity and yet not interfere with the growth of the tubercle bacillus.

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TABLE I. CULTIVATION OF TUBERCLE BACILLI FROM PATHOLOGIC MATERIAL: PREPARATION OF MEDIA

Potassium phosphate (Dihydrogen)	1.00 Gm.
Sodium phosphate (Disodium)	6.25 Gm.
Sodium citrate	1.50 Gm.
Magnesium sulfate	0.60 Gm.
"Tween" 80*	5.00 ml. of 10% aqueous solution
Casein hydrolysate	20.00 ml. of 10% neutral solution
Distilled water ad.	1,000.00 ml.

Adjust to pH 7.0-7.2 with normal sodium hydroxide
Dispense in 5 ml. volumes in properly prepared tubes
Autoclave at 15 pounds for fifteen minutes
To each 5 ml. tube of sterile media, add aseptically:
0.400 ml. of 5 per cent serum albumin in 2 per cent saline†
0.005 ml. of 10 per cent vegex,‡ autoclaved aqueous solution
0.050 ml. of 10 per cent glucose, filtered aqueous solution

*A polyoxyethylene derivative of sorbitan monooleate, product of Atlas Powder Company, Wilmington, Del. "Tween" must be kept in refrigerator and made up fresh each time medium is prepared. Stored in the mineral base it rapidly splits into products inhibitory to the growth of the tubercle bacillus.

†Bovine fraction V, Armour Laboratories, Chicago, Ill. Desiccated albumin must be kept frozen. Solution prepared fresh in 2 per cent saline for each lot of media, filtered through a Seitz or porcelain filter, and inactivated at 56° C. for thirty minutes.

‡Product of Vegex Corporation, New York, N. Y.

Mineral base may be stored at room temperature with a few drops of chloroform.

"Tween," albumin, glucose, and vegex must be added just prior to use.

All minerals C.P., not recrystallized.

The fluid medium was dispensed in 5 ml. amounts in 7 by 1 Pyrex glass test tubes which previously had been chemically cleaned, rinsed with distilled water, stoppered with cotton, and autoclaved at 15 pounds of pressure for fifteen minutes instead of the usual sterilization by dry heat. The preparation of glassware is most important, since residual soaps, oils rendered from cotton plugs by dry heat, fats, etc., may inhibit the growth of the tubercle bacillus.

Dubos[8] also has described a solid medium prepared by the addition of 1.5 per cent agar-agar and 0.001 per cent ferric ammonium citrate to the mineral solution with "Tween" (Table I). The mineral base, "Tween," and agar-agar are autoclaved; vegex, glucose, and serum albumin are added aseptically. The agar is dispensed in Petri dishes with moist filter paper attached inside the cover. After inoculation the dish is sealed with adhesive tape to prevent the loss of moisture by excessive evaporation.

For cultural examination, 5 to 20 ml. volumes of the specimens were digested for periods varying from one-half to two hours at room temperature with an equivalent volume of 3 per cent hydrochloric acid, then neutralized to approximately pH 7.0 with 3 per cent sodium hydroxide. The digest was centrifuged and the sediment concentrated in one-tenth to one-twentieth the original volume in sterile physiologic saline. The shorter periods of digestion are desirable since prolonged treatment with acid or alkali undoubtedly kills some of the tubercle bacilli in the sample. Catheterized or bladder urines and gastric aspirations usually can be freed of commensural bacteria by digestion for one-half hour or less, while material such as sputum usually requires longer periods. Media were inoculated with 0.1 to 0.5 ml. of digest and incubated at 37° C. for twenty-one days. Acid-fast smears were examined as soon as visible growth appeared; in any case, on the seventh, fourteenth, and twenty-first days of incubation. The final smear on the twenty-first day was made on the centrifuged sediment of the broth cultures.

Since the previous report,[6] 197 specimens have been examined for tubercle bacilli by these methods as well as by guinea pig inoculation. Of these, twenty-eight were positive either by culture or guinea pig, or both. The results obtained with these positive specimens are summarized in Table II.

TABLE II. CULTIVATION OF TUBERCLE BACILLI FROM PATHOLOGIC MATERIAL: COMPARISON OF GUINEA PIG INOCULATION WITH CULTIVATION IN DUBOS' MEDIA

MATERIAL	NUMBER OF POSITIVE SPECIMENS	NUMBER POSITIVE BY		INCUBATION IN DAYS	
		GUINEA PIG	CULTURE	RANGE	MEAN
Biopsy material					
Eye	1	1	1	7	
Lymph nodes	2	2	2	7	9
Miscellaneous	2	2	2	10-14	
Gastric aspiration	2	1	2*	14-21	12
Joint fluid	3	3	3	5-21	15
Urine					
Bladder	5	5	5	7-15	12
Catheterized	2	2	2	7- 9	8
Sputum	6	6	4†	7	7
Spinal fluid	5	5	3	10	10
Total	28	27	24	5-21	10.4

*Patient with positive culture and negative guinea pig not available for further study.
†One of two guinea pigs positive on each of two specimens which were negative by culture.

RESULTS

As is shown in Table II, twenty-three (82 per cent) of the twenty-eight specimens which contained tubercle bacilli were positive by culture as well as by guinea pig. One additional specimen, a gastric aspiration which was positive by culture, did not produce tuberculosis in guinea pigs. The incubation periods of these positive specimens ranged from five to twenty-one days, with a mean of 10.6 days, remarkably little time for the bacteriologic diagnosis of tuberculosis. The remaining specimens, two sputa and two spinal fluids, were positive by guinea pig only, tubercle bacilli not being found in culture.

The results obtained with this small series of specimens are combined with those previously reported[6] in Table III. Of the fifty-seven specimens which contained tubercle bacilli, fifty (87.7 per cent) were positive by culture, a figure which is not significantly different from the fifty-four (94.6 per cent) specimens which were positive in guinea pigs. The incubation periods for the positive cultures in this larger series ranged from five to twenty-seven days, with a mean of 10.6 days. Forty-four (88 per cent) of the positive results

TABLE III. CULTIVATION OF TUBERCLE BACILLI FROM PATHOLOGIC MATERIAL: RESULTS OBTAINED WITH POSITIVE SPECIMENS

MATERIAL	NUMBER OF SPECIMENS	NUMBER POSITIVE BY		PER CENT POSITIVE BY CULTURE	INCUBATION IN DAYS	
		GUINEA PIG	CULTURE		RANGE	MEAN
Biopsy, surgical, and post-mortem material	7	7	7	100.0	6-14	10.5
Gastric aspiration	8	7*	8	100.0	7-27	12.0
Joint fluid	5	5	5	100.0	5-21	14.3
Urine	14	14	13†	92.8	7-20	11.4
Sputum	16	14‡	13†	81.2	5-21	7.6
Spinal fluid	7	7	4	57.1	6-10	8.0
Total	57	54	50	87.7	5-27	10.6

*Patient with negative guinea pig and positive culture not available for further study.
†One guinea pig positive on case with negative culture.
‡Two sputa negative in guinea pigs and positive by culture were from known cases of tuberculosis.

were obtained with incubation periods of fourteen days or less, a sharp contrast to the eight to twelve weeks usually required for diagnosis by guinea pig inoculation.

As yet, too few positive specimens have been studied on the solid medium to permit analysis. The few strains isolated as well as the stock cultures of tubercle bacilli grew as small, smooth, soft, colorless, semitransparent colonies after seven to ten days of incubation. Colony morphology was not unlike that of the streptococcus, markedly different from the hard, granular, opaque growth usually observed in coagulated egg media. Four spinal fluids which failed to grow in broth yielded positive results within ten days when cultivated on the agar medium.

DISCUSSION

The specimens (Table IV) with which divergent cultural and guinea pig results were obtained are of particular interest. A positive cultural result with those specimens which were negative by guinea pig suggests either of two explanations: such results merely represent differences in the number of tubercle bacilli in the inocula used for culture and guinea pig or these strains were avirulent. In the case of the two sputa, the former seems the most likely since both specimens were obtained from patients known to have tuberculosis. In the case of the gastric aspiration, repeat specimens for further study were unobtainable and it could not be established whether the patient did or did not have tuberculosis. The microorganism isolated had the cultural and growth characteristics of tubercle bacilli but was avirulent for guinea pigs.

TABLE IV. CULTIVATION OF TUBERCLE BACILLI FROM PATHOLOGIC MATERIAL: DISCREPANT RESULTS BETWEEN GUINEA PIG INOCULATION AND CULTIVATION IN DUBOS' MEDIA

MATERIAL	TOTAL NUMBER OF SPECIMENS	NUMBER OF SPECIMENS		
		POSITIVE G.P. POSITIVE CULTURE	POSITIVE G.P. NEGATIVE CULTURE	NEGATIVE G.P. POSITIVE CULTURE
Gastric aspiration	8	7	0	1
Urine	14	13	1	0
Sputum	16	11	3	2
Spinal fluid	7	4	3	0

A positive guinea pig and a negative culture on the same specimen also is amenable to either of two explanations. If the material contained but a few tubercle bacilli, it is conceivable that acid digestion might reduce the number of viable cells below the minimum necessary for growth in artificial media. The alternate explanation is the probability that certain strains fail to grow in these media. Dubos[8] feels that the medium does not yet provide the optimal factors essential for the growth of all strains of tubercle bacilli. That there are differences between various strains is well illustrated by the four spinal fluids which were negative in fluid media but yielded prompt positive results when cultured on Dubos' agar.

Saprophytic Mycobacteria as yet cannot be sufficiently differentiated to permit the exclusive use of these media for the specific diagnosis of tuber-

culosis on certain kinds of material; namely, urine and gastric aspiration. However, in those instances where further bacteriologic identification is indicated, the use of an isolated culture for guinea pig injection affords a considerable saving of time.

SUMMARY

The fluid and solid media recently described by Dubos have been successfully applied to the rapid cultivation of tubercle bacilli from pathologic material.

Of fifty-seven positive specimens examined in fluid media, fifty (87.7 per cent) were positive by culture, while fifty-four (94.6 per cent) were positive by guinea pig inoculation. Incubation periods for the positive cultures ranged from five to twenty-seven days, with a mean of 10.6 days. Forty-four (88 per cent) of the positive results were obtained within fourteen days of incubation.

Too few specimens have been examined on the solid medium to permit analysis. Four strains which failed to grow in broth grew readily on the solid medium. Colony morphology on this medium is strikingly different from that observed on coagulated egg media. Growth occurs as soft, discrete, semitransparent colonies not unlike those of the streptococci.

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DISCREPANCIES IN THE AGGLUTINATION TEST FOR BRUCELLOSIS AS PERFORMED WITH VARIOUS ANTIGENS AND AS REPORTED FROM DIFFERENT LABORATORIES

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INCONSISTENCIES in the results of brucella agglutination tests when performed on the same serum with different antigens have been the cause of concern in our laboratory. Although close attention was given to uniformity and accuracy of the technical details of the test, nevertheless, significant differences in titer have been observed with disconcerting frequency when several commercial antigens were used in routine testing. Furthermore, results obtained with different lots of the same commercial antigen were not infrequently at variance with each other.

Similar experiences have been noted by others. Serum from a patient with culturally proved chronic brucellosis reported by Spink, Hall, and Aagaard[1] was sent to two different laboratories for agglutination tests. One laboratory reported that no brucella agglutinins were present; the other reported an agglutinin titer of 1:400. Angle, Algie, and Morgan[2] observed similar discrepancies and cite an example in which a serum was reported by a state laboratory as giving a negative test for brucella agglutinins, but on which positive tests were obtained by them in dilutions of 1:640 with three commercial antigens.

The present study was undertaken to explore the extent of the variability of brucella agglutinin titers as determined with different antigens and also as reported by different laboratories. It is an attempt to determine the deviations one might expect to encounter in actual practice under existing laboratory conditions.

PROCEDURE

Fifteen milliliters of serum were collected from each of eighteen individuals known to have moderate brucella agglutinin titers. In seven of the patients, blood cultures had been positive for *Brucella abortus* from sixteen months to seven years before, but the infection was considered to be quiescent at the time of the present tests. In one individual, brucella agglutinins had developed in response to vaccination for cholera.[3] The other ten were presumed either to be suffering from chronic brucellosis or to have recovered from the disease. In addition, sera were collected from three rabbits, each of which had been infected with one of the three species of *Brucella—melitensis, abortus,* and *suis.* Because of the very high agglutinin titers of these rabbit sera, dilutions were made with normal rabbit serum to bring the titers into a more moderate range.

The opsonocytophagic test was done on the blood of each patient at the time the serum was collected. In the blood of fifteen of the patients, there was marked phagocytosis by 80 per cent or more of the leucocytes, while the other three showed moderate phagocytosis. This tends to confirm further the specific identity of the antibody response in these patients.

Each serum was collected and prepared aseptically and was divided into six lots in sterile, stoppered tubes. One tube of each serum was sent to each of five laboratories other

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TABLE I. THE RESULTS OF BRUCELLA AGGLUTINATION TESTS PERFORMED ON

SERUM	TESTS IN OUR LABORATORY									
	TEST TUBE ANTIGENS			RAPID SLIDE ANTIGENS						
	1	2	3	4	5	6	7	8	9	10
1. N. C.	1/160	1/320	1/160	1/160	1/200*	1/100	1/400*	1/200	1/100	1/200
2. M. A.	1/160	1/320	1/160	1/160	1/200*	1/100†	1/200	1/200	1/100	1/200†
3. D. S.	1/160	1/160	1/80	1/160	1/200*	1/100	1/400†	1/100	1/100	1/100
4. S. L.	1/160	1/320	1/320	1/160	1/200*	1/200†	1/400*	1/200	1/100	1/200†
5. S. B.	1/80	1/160	1/80	1/80	1/100	1/50	1/100	1/100	1/50	1/50
6. M. A.	1/320†	1/320	1/160	1/160†	1/100	1/100†	1/200	1/100	1/50	1/100
7. G. F.	1/80	1/160	1/80	1/160	1/100	1/100	1/400*	1/200†	1/50	1/50
8. J. B.	1/1280			1/320*	1/200*	1/500*	1/400*	1/400*	1/400*	1/400*
9. C. C.	1/320†	1/640	1/320	1/320*	1/200*	1/200†	1/400*	1/200	1/100	1/100
10. A. B.	1/40	1/80	1/40	1/80	1/50	1/25	1/100	1/50†	1/50	1/50
11. W. F.	1/320†	1/320		1/160	1/200*	1/100	1/400*	1/200	1/100	1/200
12. M. F.	1/80	1/160	1/80	1/80	1/50	1/25	1/100	1/50	1/50	1/50
13. P. M.	1/160	1/640	1/160	1/160	1/200*	1/100	1/400*	1/100	1/50	1/100
14. J. S.	1/640†	1/1280	1/640	1/160	1/200*	1/100	1/400*	1/200	1/100	1/200†
15. W. T.	1/320	1/1280	1/640	1/320*	1/100	1/100	1/400*	1/200	1/50	1/100
16. P. V.	1/320	1/640	1/320	1/160	1/200*	1/100	1/400*	1/200†	1/100	1/200
17. J. W.	1/20			1/40	1/25†	1/25	1/100	1/25	Neg.	1/25
18. J. G.	1/160		1/160	1/160†	1/100	1/50†	1/200	1/100	1/100	1/200†
19. Rabbit—Br. abortus	1/640				1/200*		1/400*	1/400*	1/100	1/500
20. Rabbit—Br. melitensis	1/640				1/200*		1/400*	1/50	1/50	1/100
21. Rabbit—Br. suis	1/320				1/200*		1/400*	1/100	1/100	1/200

*The maximum titer obtainable with this antigen.
†Partial agglutination but agglutination usually complete in the next lower dilution.

than our own with a request for routine brucella agglutination tests. No information was given to the laboratory concerning the clinical histories, nor were the reasons for requesting these tests divulged. Two public health laboratories, two private clinical laboratories, and one veterinary laboratory were selected as representative of those commonly used in practice.

The sixth tube of each serum was tested in our own laboratory against the following ten antigens: seven commercial rapid slide antigens, one commercial test tube antigen, one test tube antigen supplied by the Bureau of Animal Industry of the U. S. Department of Agriculture, and one test tube antigen prepared in our laboratory from *Br. abortus* strain 295 in accordance with the directions of Huddleson.[5] The eight commercial antigens were purchased on the open market and represented all those which were available to us after a thorough search of sources. Several of the specimens were not tested with all the antigens because of insufficient sera. All tests performed in our laboratory were in accordance with standard techniques.[7]

As a matter of interest, sera were similarly collected from three patients who had recently received intravenous combined typhoid vaccine for fever therapy. These sera were tested for typhoid agglutinins in our laboratory and were also submitted to five other laboratories. The same laboratories were used as for the brucella agglutination tests, except that a hospital laboratory was substituted for the veterinary laboratory.

RESULTS

The results of the brucella agglutination tests performed in our laboratory as well as those reported from the five other laboratories are presented in Table I. The table also gives the maximum and minimum values reported for each serum by the five laboratories as well as the maximum and minimum values of all fifteen tests.

THE SAME SERUM WITH DIFFERENT ANTIGENS AND IN DIFFERENT LABORATORIES

TESTS IN OTHER LABORATORIES					RANGE OF ALL 15 TESTS		RANGE OF REPORTS FROM OTHER LABORATORIES	
PUBLIC HEALTH LABORATORY	PUBLIC HEALTH LABORATORY	PRIVATE CLINICAL LABORATORY	PRIVATE CLINICAL LABORATORY	VETERINARY LABORATORY	MAXIMUM	MINIMUM	MAXIMUM	MINIMUM
A	B	C	D	E				
1/160	1/160	1/200	1/80	1/320	1/400	1/80	1/320	1/80
1/640	1/160	Neg.	1/40	Neg.	1/640	Neg.	1/640	Neg.
1/320	1/80	Neg.	Neg.	Neg.	1/400†	Neg.	1/320	Neg.
1/160	Neg.	1/200	1/80	1/80	1/400	Neg.	1/200	Neg.
1/160	Neg.	Neg.	1/40†	Neg.	1/160	Neg.	1/160	Neg.
1/320	1/160	Neg.	1/80	Neg.	1/320	Neg.	1/320	Neg.
1/160	Neg.	1/200	1/40	1/80	1/400	Neg.	1/200	Neg.
1/1280	1/1280	1/500*		1/320	1/1280	1/320	1/1280	1/320
1/320	1/80	1/200	Neg.	1/160	1/640	Neg.	1/320	Neg.
Neg.	Neg.	Neg.	1/200	Neg.	1/200	Neg.	1/200	Neg.
1/640	1/160	1/100	Neg.	1/40	1/640	Neg.	1/640	Neg.
Neg.	Neg.	1/100	Neg.	Neg.	1/100	Neg.	1/100	Neg.
1/320	Neg.	1/100	Neg.	1/40	1/640	Neg.	1/320	Neg.
1/640	Neg.	1/200	Neg.	1/80	1/1280	1/20	1/640	Neg.
1/160	1/80	1/200	1/20	1/80	1/1280	Neg.	1/200	1/20
1/640	1/160	1/200	Neg.	1/80	1/640	Neg.	1/640	Neg.
Neg.	Neg.	Neg.	Neg.	Neg.	1/100	Neg.	Neg.	Neg.
1/50	Neg.	1/100	1/20		1/200	Neg.	1/100	Neg.
1/640	1/320	1/500*	Neg.		1/640	Neg.	1/640	Neg.
1/640	1/320	1/100	Neg.	Neg.	1/640	Neg.	1/640	Neg.
	1/320	1/500*	Neg.	1/80	1/500	Neg.	1/500	Neg.

In the titers reported from the five laboratories other than our own, it will be noted that of the twenty-one sera submitted, eighteen were called negative by one or more laboratories. Of these eighteen "negative sera," all except one were reported by other laboratories as positive in titers ranging from 1:100 to 1:640.

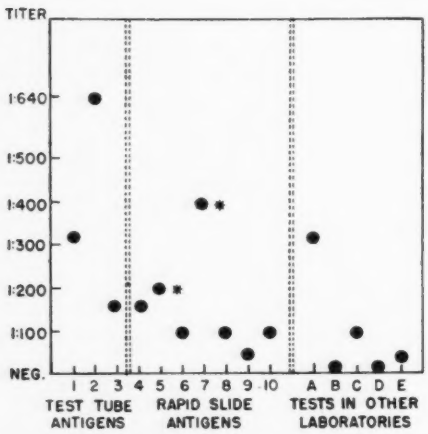
The variations of the agglutinin titers obtained in our own laboratory with the ten different antigens confirmed, in a general way, and, in a few cases, extended the magnitude of the deviations reported by the other laboratories. In our laboratory, however, only one serum was found to be negative in any test.

The variations in the brucella agglutination tests of six selected cases are presented graphically in Fig. 1.

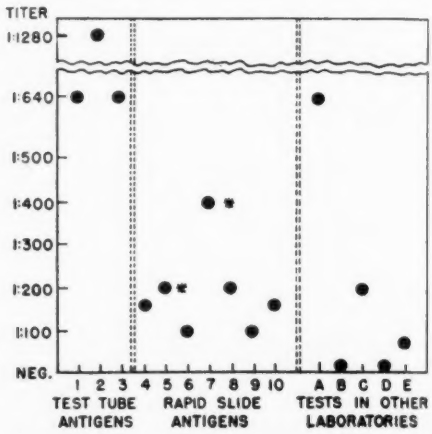
The typhoid and paratyphoid agglutination tests that were reported on the sera from three patients treated with intravenous typhoid combined vaccine are presented in Table II. Inconsistencies in the titers reported by different laboratories are evident in these data also.

DISCUSSION

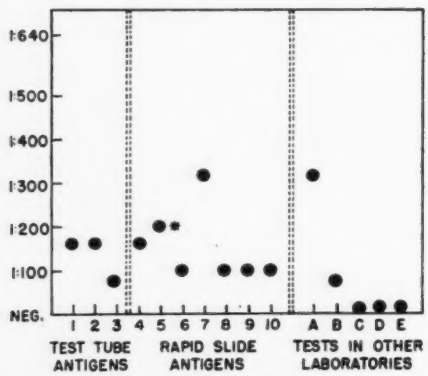
The brucella agglutination test is more widely used than any other procedure in the diagnosis of brucellosis. It is used extensively in the diagnosis of the human disease as well as being the generally accepted criterion for the presence of infection in cattle. At the first Inter-American Brucellosis Congress held in Mexico City in 1946, various speakers indicated that "the agglu-



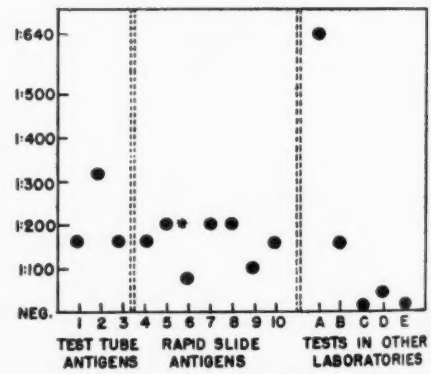
CASE P.M. ACUTE BRUCELLOSIS WITH BLOOD CULTURES POSITIVE FOR BR. ABORTUS 26 MONTHS BEFORE. PROMPT AND COMPLETE CLINICAL RECOVERY WITHOUT RELAPSE.



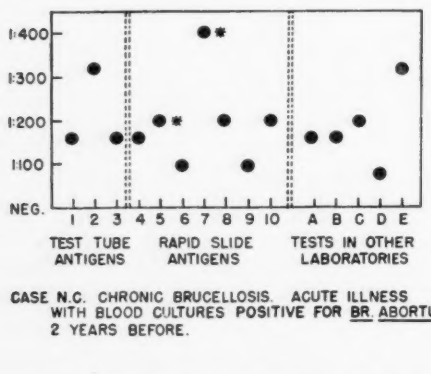
CASE J.S. ACUTE BRUCELLOSIS WITH BLOOD CULTURES POSITIVE FOR BR. ABORTUS 28 MONTHS BEFORE. PROMPT AND COMPLETE CLINICAL RECOVERY WITHOUT RELAPSE.



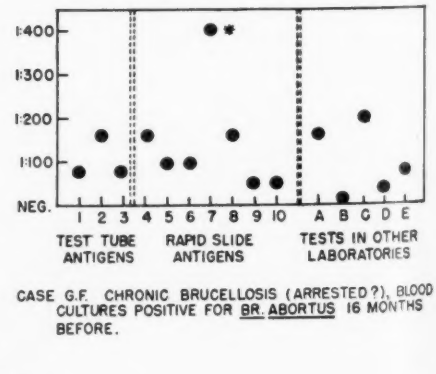
CASE D.S. "PROBABLE" CHRONIC BRUCELLOSIS



CASE M.A. "PROBABLE" CHRONIC BRUCELLOSIS



CASE N.C. CHRONIC BRUCELLOSIS. ACUTE ILLNESS WITH BLOOD CULTURES POSITIVE FOR BR. ABORTUS 2 YEARS BEFORE.



CASE G.F. CHRONIC BRUCELLOSIS (ARRESTED?), BLOOD CULTURES POSITIVE FOR BR. ABORTUS 16 MONTHS BEFORE.

* THE MAXIMUM TITER OBTAINABLE WITH THIS ANTIGEN

Fig. 1.—Variations in the results of brucella agglutination tests on the same serum when performed with various antigens and when reported from different laboratories.

TABLE II. AGGLUTINATION TESTS FOR TYPHOID AND PARATYPHOID: RESULTS OF TESTS ON THE SAME SERUM REPORTED FROM DIFFERENT LABORATORIES

		OUR LABORATORY		OTHER LABORATORIES				
PATIENT	ANTIGEN	TEST TUBE ANTIGEN	PLATE ANTIGEN	PUBLIC HEALTH LABORATORY A	PUBLIC HEALTH LABORATORY B	PRIVATE CLINICAL LABORATORY C	PRIVATE CLINICAL LABORATORY D	HOSPITAL LABORATORY F
1 (A. F.)	Typhoid		1/320*	Neg.			1/500*	
	Typhoid O				Neg.	1/400		1/640
	Typhoid H				1/1280	1/1000		1/1280
	Paratyphoid A				Neg.	1/500		1/2560
	Paratyphoid B				1/640	1/500		1/2560
2 (V. F.)	Typhoid	1/100	1/320*	1/160				
	Typhoid O	1/50			Neg.	1/200	1/80	1/640
	Typhoid H	1/250			1/80	1/400	1/160	1/640
	Paratyphoid A				1/160	1/200	1/160	1/640
	Paratyphoid B				1/320	1/400	1/160	1/640
3 (H. W.)	Typhoid	1/2500	1/320*	1/640				
	Typhoid O	1/1000			Neg.	1/300	1/640	1/2560
	Typhoid H	1/1000			1/320	1/2000	1/320	1/2560
	Paratyphoid A				1/1280	Neg.	1/320	1/2560
	Paratyphoid B				1/1280	Neg.	1/640	1/2560

*The maximum titer obtainable with this antigen.

tination test continues to be the most useful procedure in the diagnosis of active brucellosis.''[4] It is therefore of considerable importance to evaluate the reliability and accuracy of the test as it is applied in actual practice.

The significance of the agglutination test in relation to human brucellosis has long been subject to discussion, and its interpretation in terms of clinical infection may be a difficult task indeed. The question is frequently asked, what is a clinically significant titer? An unqualified answer cannot be given and a number of limitations must be considered.

Several sources of difficulty in the interpretation of the brucella agglutination test arise from biologic factors. It is well known that individuals vary markedly in their ability to produce brucella agglutinins in response to clinical infection. Also, different strains of the organism vary in their agglutinogenic power. Many authors[1, 2, 5] have reported cases of brucellosis verified by blood cultures in which no serum agglutinins appeared. Conversely, apparently healthy individuals with significant titers in their blood are found in brucella-agglutinin surveys. Thus, blood from a patient who has brucellosis may contain no agglutinins, while blood from a symptomless individual may contain them.

It has long been known that infection with *Pasturella tularense* produces a cross-agglutination response for brucella. Recently, we have shown that significant titers of brucella agglutinins, as well as opsonins, develop in most individuals receiving the standard two-dose cholera vaccination.[3] This response is due to an H antigen of *Vibrio comma.*

In addition to the irregularities of agglutinin response arising from the uncontrollable biologic variables, the present studies demonstrate that the measurement of brucella agglutinins in any given serum is subject to great variation with the usual laboratory methods. The magnitude of this variation is such that of twenty-one sera submitted to five qualified laboratories for routine test-

ing, reports on each of eighteen varied from completely negative to positive in dilutions of 1:100 to 1:640. Two-thirds of the so-called "negative tests" were reported elsewhere as positive in dilutions of 1:320 or 1:640—titers which are considered by many physicians as substantiating a clinical diagnosis of brucellosis. Similarly, a negative test is often regarded as excluding that diagnosis. Depending on which laboratory report is accepted as the actual titer, at least two-thirds of the cases of this series can be thus either "ruled out" or "substantiated." That such blind reliance on agglutination tests is deplorable is well demonstrated by these data. Our views on the clinical interpretation of the agglutination test have been discussed in previous publications.[6]

We do not intend to imply that the brucella agglutination test is a useless procedure. If one is not indifferent to the sources of error and if one does not attempt to interpret the test too strictly, it may be a useful adjunct to diagnosis. We believe that the variations observed in this study are mainly due to a lack of standardization of the antigens. Angle, Algie, and Morgan[2] noted similar diversity in agglutination titers when they used antigens which they prepared from twenty stock strains of brucella. Variations of technique of the test may also be a contributing factor. The admonition of the Committee on Bang's Disease of the United States Live Stock Sanitary Association[7] bears repeating: "The reading of tests should be carried on only by individuals who have had wide experience in this work. Extreme care should be exercised in the observation of tests to minimize the discrepancies resulting from improper or careless observation." It would appear that much of the difficulty could be avoided if standardization of all antigens were supervised by some central agency, or if standard sera were available for checking antigens. The test as used in our laboratory has given consistent results when antigens prepared from the same strain have been employed.

Limited observations on the results of typhoid and paratyphoid agglutination tests made on sera from patients treated with typhoid vaccine suggest that the variations observed in the agglutination test for brucella also occur in other agglutination tests. It is noteworthy that the agglutinin response in these patients was the result of vaccine therapy rather than of a natural infection, as was the case in the brucella sera. It should also be mentioned that inconsistencies in the testing of typhoid and paratyphoid agglutinins are of considerably less clinical importance than in the case of brucella agglutinins. In comparison with brucellosis, the agglutination test plays a relatively minor role in the diagnosis of the enteric infections.

SUMMARY

1. Wide variations in brucella agglutinin titers were observed in each of twenty-one sera tested with ten different antigens in our laboratory. Tests on the same sera made in five other laboratories showed equally great discrepancies.

2. Of the twenty-one sera tested, eighteen were called negative by one or more laboratories. Of these eighteen "negative sera," sixteen, or 89 per cent, were elsewhere called positive in titers of 1:200 or higher; twelve, or

67 per cent, were called positive in dilutions of 1:320 or 1:640—titers which are frequently considered as substantiating a clinical diagnosis of brucellosis.

3. Depending on which laboratory report is accepted as the actual titer, two-thirds of the eighteen cases can be either "ruled out" or "substantiated" by commonly accepted but deplorable practices of interpretation of the test.

4. Limited observations on typhoid and paratyphoid agglutination tests suggest that the variations observed in the tests for brucella also occur in other tests.

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HUMAN BRUCELLA MELITENSIS INFECTIONS IN MINNESOTA WITH HOGS AS THE PROBABLE SOURCE

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MINNEAPOLIS, MINN.

THREE species of the genus *Brucella, abortus, melitensis,* and *suis,* are recognized as the etiologic agents causing brucellosis in man and animals. *Brucella abortus* and *Brucella suis,* etiologic agents for brucellosis in cattle and hogs, respectively, have been recognized in Minnesota since laboratory diagnosis was begun. The *melitensis* species, usual incitant of brucellosis in sheep and goats, has not previously been established as present in man or livestock in this state.

Kabler and MacLanahan[1] in 1936 reported on forty strains of Brucella isolated in Minnesota from human cases of brucellosis. They classified these strains as follows: twenty-five strains of *Br. suis,* thirteen strains of *Br. abortus,* and two strains with conflicting reactions, one of which appeared to be *Br. suis* and the other *Br. melitensis.*

Jordan and Borts[2] in 1946 reported the isolation of twenty-six strains of *Br. melitensis* in Iowa from human cases of brucellosis. One strain was isolated in 1930, and twenty-five strains have been isolated since December, 1943.

The present report, based on laboratory findings and epidemiologic investigations made in 1945-1946 by the Minnesota Department of Health, deals primarily with thirteen strains of *Br. melitensis* isolated from human cases of brucellosis.

MATERIALS AND METHODS

Blood Culture.—Twenty-five milliliters of bacto-tryptose broth, pH 6.8, containing 1 per cent sodium citrate, were placed in a 2-ounce rubber diaphragm screw-capped bottle and sterilized. A lead foil cover was placed over the cap and crimped tightly around the neck of the bottle. The bottle was then mailed to the physician with the instructions to draw, aseptically, 5 ml. of blood from the patient during a febrile attack, to insert the needle through the diaphragm of the cap and expel the blood from the syringe into the broth, replace the lead foil over the cap of the bottle, and return the bottle to the laboratory by mail.

Upon receipt of the culture in the laboratory, approximately 10 per cent of the air was aseptically removed from the bottle and replaced with an equal volume of CO_2. The broth culture was then incubated at 37° C. After five days of incubation, four tubes of bacto-tryptose agar, pH 6.8, were inoculated, each tube receiving approximately 1 ml. of broth culture. Two tubes were incubated aerobically at 37° C. and two tubes in an atmosphere of 10 per cent CO_2. The broth culture was returned to the 37° C. incubator for another ten days, after which time four more tryptose agar slants were inoculated and incubated as previously described. Each set of subcultures was observed every other day for a period of two weeks. No blood cultures were discarded as negative before thirty days.

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*Section of Medical Laboratories.
†Section of Preventable Diseases.

Brucella species were identified according to their CO_2 requirements, staining reactions, fermentation reactions, bacteriostatic action of dye plates, H_2S formation as described by Huddleson and co-workers,[3] and serologic tests.

Agglutinations.—Macroscopic tube agglutination tests for the presence of Brucella agglutinins were made with serum obtained from clotted whole blood specimens submitted by the attending physician.

LABORATORY AND EPIDEMIOLOGIC DATA

During the years of 1945 and 1946, a total of 193 Brucella strains were recovered from human sources. Of these strains, 165 (84.5 per cent) were *Br. abortus*, 15 (7.8 per cent) were *Br. suis*, and 13 (6.7 per cent) were *Br. melitensis*.

The results of the laboratory tests and the epidemiologic findings of the thirteen human cases of brucellosis due to *Br. melitensis* are summarized in Table I.

TABLE I. THIRTEEN CASES OF HUMAN BRUCELLOSIS DUE TO *Br. melitensis*: SUMMARY OF LABORATORY FINDINGS AND EPIDEMIOLOGIC DATA

CASE	INITIALS	AGE	AGGLUTINATION REACTION		POSITIVE BLOOD CULTURES	ANIMAL CONTACT		PROBABLE SOURCE OF INFECTION
			DATE RECEIVED	TITER	DATE RECEIVED	HOGS	CATTLE	
1	G. D.	53	2/13/45	1:2560	2/16/45	Yes	No	Packing plant, handling hog salvage
2	C. H.	49	3/26/45	1:2560	3/28/45	Yes	No	Packing plant, handling hog casings
3	W. F.	40	3/31/45	1:320	4/ 3/45	Yes	No	Packing plant, handling hog casings
4	M. P.	19	4/30/45	1:5120	5/23/45	Yes	No	Packing plant, handling hog casings
5	W. K.	50	5/10/45	1:1280	5/13/45	Yes	No	Packing plant, lard making
6	K. D.	29	6/ 8/45	1:5120	6/21/45	Yes	No	Packing plant, hog kill
7	R. B.	20	6/21/45	1:1280	6/24/45	Yes	No	Packing plant, hog kill
8	L. J.	29	7/13/45	1:640	7/20/45	Yes	No	Packing plant, pork trim
9	R. C.	25	9/11/45	1:160	9/20/45	Yes	No	Packing plant, shackling hogs
10	C. H.	34	5/ 2/46	1:5120	5/15/46	Yes	Yes	Farm abortion in hogs (cows nonreactors)
11	I. S.	51	6/19/46	1:2560	6/21/46	Yes	No	Packing plant, hog kill
12	L. H.	30	7/ 6/46	1:5120	7/14/46	Yes	Yes	Farm, handling aborted material from hogs (no history of abortion in cattle)
13	R. D.	35	7/30/46	1:5120	8/ 3/46	Yes	No	Packing plant, shackling hogs

All the cases presented in Table I are men; the youngest was 19 years of age, and the oldest 53 years. All had symptoms consistent with the diagnosis of brucellosis. Eleven of the thirteen patients were packing plant employees who handled either live hogs or pork products. Six of the employees handled the hogs at the time of killing, and four handled the pork within forty-five minutes of the killing. One employee, a pork trimmer, handled the pork the day after the animal was killed.

It is noteworthy that all the thirteen patients were residents of four Minnesota counties that border the northern section of Iowa. Two were farmers,

both of whom owned hogs bred and raised locally which aborted. There was no history of abortion in the cattle on either of these farms. Unpasteurized cow's milk was used by two of the thirteen patients; and one obtained raw milk from a cow that gave a negative reaction when tested for Bang's disease.

The hogs handled at the packing plant all came from Minnesota or from points in Iowa, and North and South Dakota, which are readily accessible by truck and rail to the Minnesota market.

COMMENTS

The isolation of *Br. melitensis* from the blood of persons in southern Minnesota demonstrates this organism as being endemic in this area.

Br. melitensis was originally regarded as the etiologic agent of brucellosis in sheep and goats, and it was believed that these animals act as reservoirs for this organism; however, there is evidence[4, 5] that all species of Brucella may infect cows, hogs, sheep, or goats. That hogs are susceptible to *Br. melitensis* is indicated in the statement by Huddleson[5]: "Melitensis is pathogenic for the hog. While I was in Malta in 1938, I saw evidence of its pathogenicity. A considerable amount of left-over unpasteurized goat's milk was fed to a group of pregnant sows. All of them aborted. *Melitensis* was recovered from the fetuses. For some reason this observation was never reported in the literature."

In the series of cases presented in this report, there is no history of contact with sheep or goats. The epidemiologic findings show that all patients had direct contact with hogs; in two cases there was contact with cattle in which there was no history of Bang's disease.

SUMMARY

1. *Br. melitensis* has been isolated from blood cultures of thirteen human cases of brucellosis.

2. All the patients were men having direct contact with hogs; eleven were packing plant employees and two were farmers.

3. Epidemiologic studies of *Br. melitensis* infections incriminate hogs as the source of infection; in at least two of the cases, the hogs were bred and raised in Minnesota.

4. All human cases of brucellosis due to *Br. melitensis* that have been detected to date are residents of four counties in south-central Minnesota.

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THE DIFFRACTION METHOD OF MEASURING RED BLOOD CELLS

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THE history of the diffraction method of measuring red blood cells is a story of a lucky accident. It is only because of a mistake by a technical assistant that the method is in use today and has been able to make its contribution to medical science both by stimulating investigations into the diameters of red cells and as a diagnostic method. In 1918 I planted a drop of centrifuged urine into clear broth. Next morning there was a faint haziness, indicating bacterial growth, but on shaking the bottle, a small number of glittering small particles became detached from the bottom. They whirled through the liquid and exhibited a bluish-greenish radiancy. Thinking I had found a new color-producing microbe, I made subcultures on agar. A variety of colonies developed, none of them obviously colored. One evening, having another look at the colonies, I moved the Petri dish up and down in front of an electric light bulb. It was then that I noticed that some colonies exhibited a curious colored gloss, in which bluish green predominated, with a faint suggestion of the other colors of the spectrum. (This phenomenon, once one knows of it, can easily be seen in thin surface cultures of many bacteria if one holds the culture between the eye and a bright source of light, preferably in a darkroom. It is what textbooks sometimes vaguely call "iridescence.") These colonies turned out to be ordinary staphylococci, but since the color phenomenon persisted in subcultures, I started making extracts of them with various solvents, hoping to isolate the elusive coloring substance. I failed in this, and then it dawned upon me that the phenomenon might be physical and not chemical. I first thought of fluorescence and then suddenly realized that I was dealing with diffraction of light, the surface culture acting as a diffraction grating. I went back to the original broth culture and found that the shiny particles were powdered glass and had nothing to do with the phenomenon, having merely found their way into the broth through a mistake of a technical assistant. (We had been using powdered glass for estimations of atmospheric bacteria, following a method then in vogue.) In pursuance of the idea that diffraction was the cause of the phenomenon, an apparatus was constructed in which a beam of white light was sent through a surface culture of staphylococci, and the resulting diffraction colors were collected and focused onto a screen.[19] A formula was developed enabling one to calculate the diameter of the cocci from the diameter of the diffraction spectra. By substituting a culture of round monilias for the cocci, a check was obtained on the correctness of the formula.[19] I then considered that the principle of diffraction, which worked so well with round cocci and round monilias, should also work with round red blood cells, and so I applied it to red blood cells.[20-22] It is shown in Fig. 1

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how a beam of white light, sent through thin end of a blood film, undergoes diffraction at the edges of the red cells and produces sets of colored rings, arranged in rainbow or spectrum fashion and sometimes called halos. This is the origin of all diffraction methods for measuring red blood cells now in use.*

It is shown in Fig. 1, and similarly in Figs. 2 and 3, that there is only one "complete" spectrum where all the colors are easily discernible. This is the spectrum to be used for measurements and comparison.

In the following years little scientific notice was taken of this method, except by Bergansius,[2] Millar,[18] Ponder,[35, 36] and Allen and Ponder.[1] During this time the technique was improved and applied to the measurement of various bacteria.[24-26, 28] It was not until 1924 that, stimulated by a publication of Hurst[15] on anemias, I applied the method to the differential diagnosis of anemias[23] and to their follow-up.[29] Various authors and firms then produced a variety of instruments for applying the method. Emmons[7] brought out the clinical eriometer. Eve[8] built a halometer which became strangely popular and which was followed by the direct halometer to which a Dr. Fine's name was attached. Pryce[42] simplified the halo method; Edwards[6] built a simple box of his own; Menzies[17] used a new refractometer (sic) designed by Dr. Malloy; Renshaw and associates[43] spoke of a hemovisor they were planning; and Bock[4] introduced the erythrocytometer which became widely used on the continent of Europe. Fullerton and co-workers[10] used an apparatus of their own and also a Ewles halometer. Christophers and Craighead[5] suggested an ingenious micromethod which employed a microscope, and Falisi[9] on similar lines produced a microdiffractometer that was followed again by Smith[45] who introduced a diffractometer manufactured by the Spencer Lens Company which appears to work well, although in common with all the methods and apparatuses mentioned, it does not allow direct comparison of two blood films. Schalm[44] had a pocket apparatus built by Zeiss; Hogben[14] constructed an apparatus of his own; and finally there is Haden's[13] Haden-Hausser erythrocytometer. This list is probably not complete; it is likely that there are further variations of the same theme still unknown to me.

An improvement in technique came in 1929 when comparison was made in one apparatus of the diffraction patterns of two different blood films.[30, 31] This was done by cutting off one half of the set of colored circles produced by each of the two films and making the remaining semicircles share the same middle-line on the receiving screen. The arrangement in diagrammatic fashion is shown in Fig. 2. This allows direct comparison of an "abnormal" with a "normal" blood film, thus doing away with the need for actual measurement in many cases, simple inspection of the two diffraction patterns being sufficient to get the required information. This design also provides for direct comparison of two blood films taken from the same patient at different times, a very useful feature in the follow-up of patients. An apparatus on these lines can easily be put together by a technical assistant. Eventually this improved technique was em-

*It was later brought to my notice that Young[50] in 1813 applied the same principle to the measurement of wool fibers (eriometer) and later of red blood cells. His work was never followed up.

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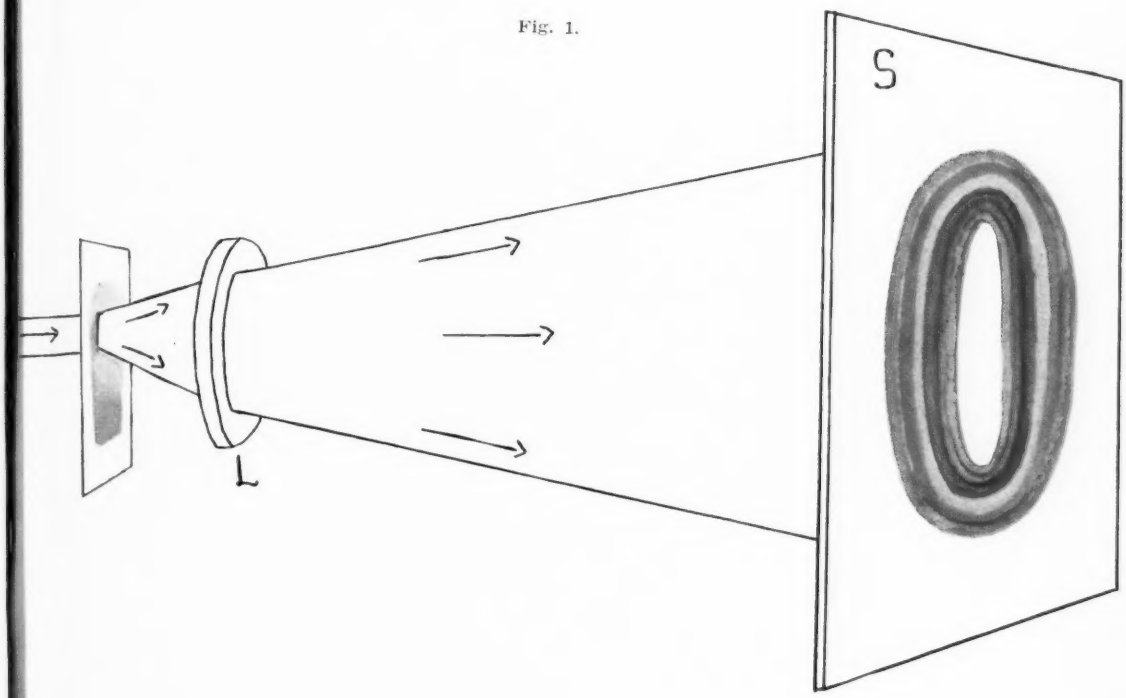
Fig. 1.



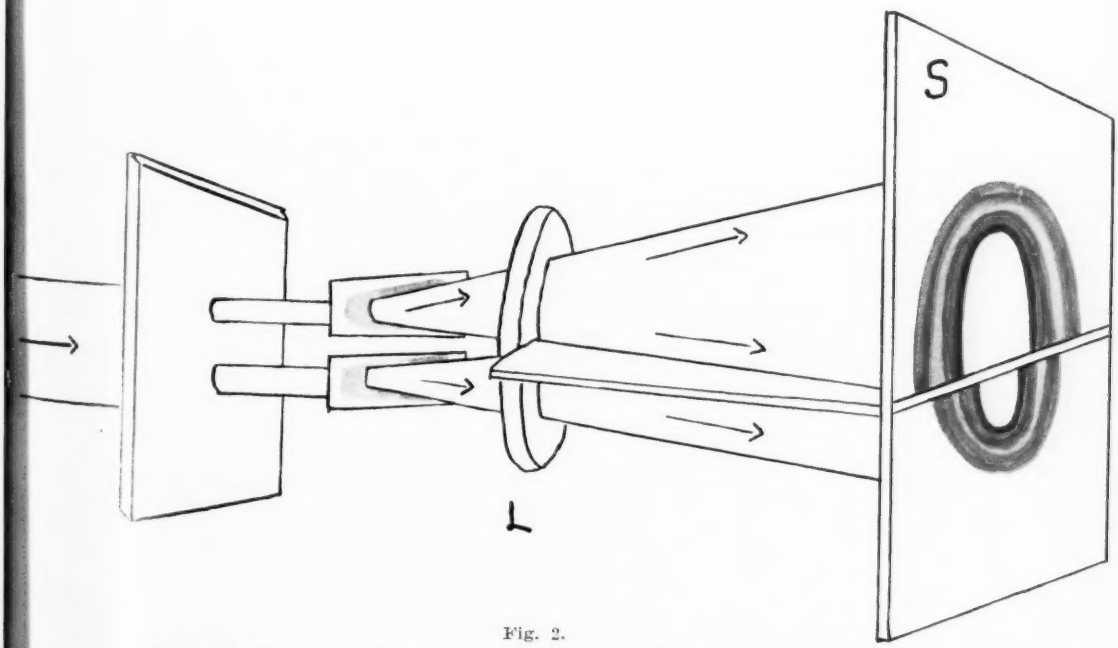


Fig. 2.

Fig. 1.—Diagram of blood film serving as diffraction grating.

Fig. 2.—Diagram of apparatus, known as Pijper's Blood Cell Tester. Direct comparison of two blood films, normal or standard blood film on one side, pernicious anemia on the other.

J. LAB. & CLIN. MED.
VOL. 32, NO. 7, JULY, 1947

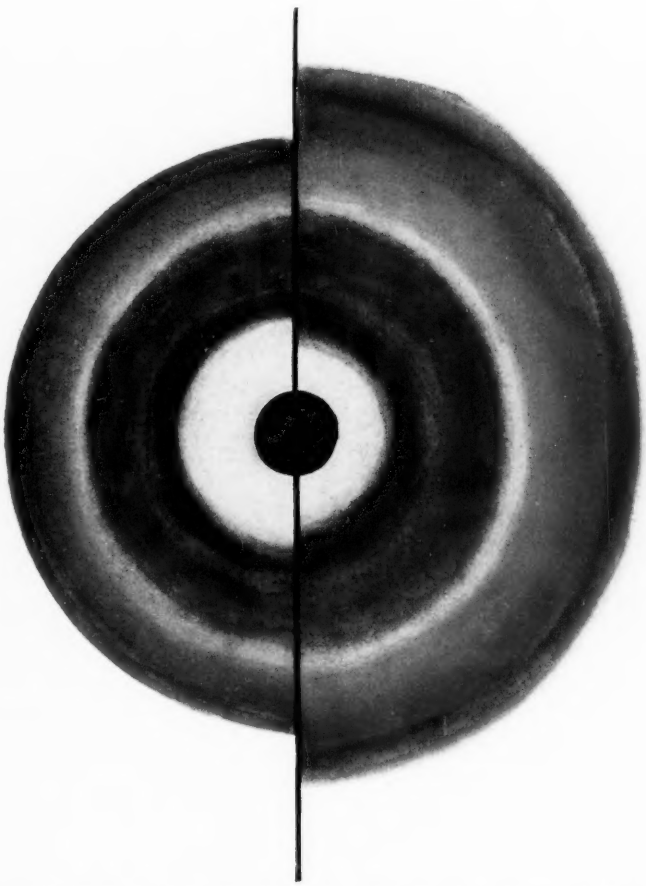


Fig. 3.—What appears on screen of apparatus when normal or standard blood is compared with anemia caused by prolonged hemorrhages, malignant growth, or similar conditions. Spectrum on right is broader, but yellow circle is not displaced. This means normal mean diameter with increased anisocytosis.

bodied in the apparatus placed on the market in 1935 by Zeiss as Pijper's Blood Cell Tester.[33, 34] Its shape and size are the result of cooperation between Prof. Siedentopf, technical adviser to the Zeiss firm, and myself. It is based on the idea illustrated in Fig. 2. Pictures of it can be found in some textbooks.[46] In Fig. 2 the spectra produced by a normal blood film and a blood film from a case of pernicious anemia are compared. In Fig. 3 is shown what actually appears on the ground-glass screen of the cell tester when normal blood and blood from a case of anemia caused by prolonged hemorrhage, malignant growth, or similar conditions are compared.

PURPOSE OF PAPER

The purpose of this paper is to show that, contrary to what is usually stated in textbooks and other publications, the diffraction method of measuring red cells, if properly applied, is capable not only of determining the mean diameter of about one million cells, but also of determining the degree and quality of anisocytosis present in a blood film. A good diffraction apparatus should allow three measurements or determinations. One gives the mean diameter, the second gives the diameter of the smallest cells present in appreciable numbers, and the third does the same for the largest cells. All this can be done in a few minutes, and in this way degree and quality of anisocytosis are determined. I do not know of any other apparatus that is capable of doing this. The technical side of this paper therefore applies to the apparatus known as Pijper's Blood Cell Tester or to any apparatus built on identical lines.*

IMPORTANCE OF DETERMINING RED CELL DIAMETERS

Modern hematology emphasizes morphology. Importance is attached to shape and size of red blood cells. Hematology owes a good deal to the careful microscopic measurements of red cells by Price-Jones and associates.[37-41] This work brought revelations, but apart from an occasional special case, Price-Jones' method and its substitutes are too elaborate for daily application.

The old-fashioned determination of the hemoglobin index as an indication of the size of the red cell still lingers on. This is indefensible because its value is arrived at by dividing a hemoglobin value by a red cell value, neither of which is usually determined with sufficient accuracy. It is admitted that by using a photoelectric colorimeter, errors in hemoglobin estimations can be very much reduced, but there is no similar reliable method yet for red cell counts. It is known that in ordinary work, with the apparatus available in most laboratories, an error of 5 per cent in both these determinations is unavoidable, an error of 10 per cent is quite common, and higher errors occur. A calculation based on two incorrect values should in principle be avoided as it leads to still more erroneous final results. What may happen, unless particular care is taken, is illustrated in Table I.

*At the present time, as far as I know, no blood cell testers can be obtained from the Zeiss firm. Perhaps an American firm will find it worth while to build an apparatus on the same lines and of identical dimensions, so that the numerical data supplied in this paper will apply.

TABLE I. ERRORS IN DETERMINATION OF HEMOGLOBIN COLOR INDEX

	HEMOGLOBIN (PER CENT)	RED CELLS	INDEX
Supposed real values	90.0	4.0	1.1
Errors of 5 per cent	85.5	4.2	1.0
Errors of 5 per cent	94.5	3.8	1.2
Errors of 10 per cent	81.0	4.4	0.9
Errors of 10 per cent	99.0	3.6	1.4

It is seen that through errors often accompanying the method, an index which in reality is 1.1 can appear as high as 1.4 or as low as 0.9. Index determinations therefore are inadvisable.

Similar objections can be raised against determinations of mean red cell volume by dividing hematocrit values by number of cells. What may happen, unless particular care is taken, is illustrated in Table II.

TABLE II. ERRORS IN DETERMINATION OF MEAN CELL VOLUME

	VOLUME OF PACKED CELLS (PER CENT)	NUMBER OF RED CELLS	MEAN CELL VOLUME
Supposed real values	39.2	3.80	103
Errors of 5 per cent	41.1	3.61	114
Errors of 5 per cent	37.3	3.99	93
Errors of 10 per cent	43.1	3.42	126
Errors of 10 per cent	35.3	4.18	84

It is seen that in a case where the mean red cell volume in reality is 103, errors often accompanying the method may lead to erroneous determinations as high as 126 or as low as 84.

There is, of course, nothing new in this criticism; many authors admit the shortcomings of these methods (Todd and Sanford[46] and Wintrobe[49]). In experienced hands the method for determination of mean red cell volume still works reasonably well, but many workers do not seem to be conscious of the fundamental fact that it is unsatisfactory to try and arrive at a correct value by dividing two incorrect values one by the other. The problem confronting hematologists nowadays is not only to place obvious cases into the correct category, but to deal with and to follow up early and mild cases in a satisfactory manner. These are the cases where deviation from the normal may be minimal, and in such cases involved calculations on not very accurate data may become misleading. There are, for example, cases of early subacute-combined degeneration of the cord, and cases of mental changes due to early pernicious anemia as described by Warburg and Jørgensen,[47, 48] where hemoglobin and red cells show too little change to lead to reliable results in the determination of either hemoglobin index or mean cell volume. There, in my experience, a direct determination of red cell diameter by the diffraction method may definitely show a quick way to the correct diagnosis.

THEORY OF DIFFRACTION METHOD

Even professional physicists admit great difficulties in attempts to give a full explanation as to what happens when a beam of light passes through a blood

film. The following formula, which applies to the conditions of Figs. 1 and 2, has, however, found favor among physicists and answers well in practice.

$$\text{Diameter red cells} = \frac{1.7\,\lambda\,\sqrt{f^2 \times r^2}}{r}$$

In this formula "λ" is the wave length of light under consideration, "f" is the focal distance of the lens (*L*) in Figs. 1 and 2 which focuses the diffraction pattern on the screen (*S*), and "r" is the radius of the colored circle of wave length "λ."

In the blood cell tester the lens has a focal distance of 20 cm., and "r" varies between 1.5 to 3.5 cm. As "r" is so much smaller than "f," the formula can be simplified by eliminating "r^2." A simple calculation will show that the average error introduced thereby is not more than 1 per cent, which is within the experimental error of the method. The formula thus becomes:

$$\text{Diameter red cells} = \frac{340{,}000\,\lambda}{r}$$

The colored circles which are of importance are those of yellow, outer edge of red, and innermost violet. This means one color rather near the middle of the spectrum and two others at the extreme ends of the spectrum. Yellow is chosen because it is a narrow band, and therefore easy to read, and quite near enough the middle of the spectrum to be suitable.

The position would be very simple if all red blood cells in a given film were of identical diameter. This is not the case in normal blood, and less so in abnormal blood. Price-Jones[37-40] has shown that red blood cells form a population and their size distribution follows a frequency curve. There are kinds of anemia where this curve is a good deal flatter than normal, in other words where the only abnormality is an increased degree of anisocytosis. Normal blood has cells of a mean diameter of about 7.5 microns, but through normal anisocytosis there is an ever-decreasing number of smaller and larger cells.

If all red blood cells in a given film were of identical diameter, it would not matter which color was chosen for applying the formula, the result would be the same in any case. The position which arises from the ever-present anisocytosis is illustrated in Fig. 4.

Shown in Fig. 4 in the left top quadrant are the three main colored circles resulting from a normal blood film as produced by diffraction by the cells of 7.5 microns, which are the most numerous ones and therefore produce the brightest colors. In a normal film the anisocytotic spread will comprise cells from 7.5 to about 6.7 microns, on the one side, and cells from 7.5 to about 8.5 microns, on the other side. In the bottom left quadrant of Fig. 4 are shown the colors produced by the largest cells, and in the top right quadrant the colors produced by the smallest cells. In actual practice of course the three sets of colored rings are superimposed, giving rise to the bottom right quadrant.

It is seen that the final position of the yellow ring is hardly affected by the superimposition of the diffraction patterns of all the red cells. It therefore

can safely be used for the calculation by means of the formula of the mean diameter of all the red cells.

It is also obvious that in the final spectrum the outer edge of the red circle is the expression of the diameter of the smallest cells in the film, and the innermost violet circle the expression of the diameter of the largest cells. These colored circles thus can be safely used in the formula for the calculation of the smallest and largest cells present. Application of the formula three times, once for yellow, once for red, and once for violet, gives the mean diameter and the smallest and largest cells, in other words, the kind and degree of anisocytosis present.

It should now be clear that any diffraction method which bases its measurements on anything but the yellow circle for the calculation of mean diameter is faulty. A measurement based on the position of the outer edge of the red circle, as is often done, will result in calculating not the mean diameter, but the diameter of the smallest cells. In normal blood this may not make much difference, as the values for mean diameter and smallest diameter lie close together. But in cases where anisocytosis is increased, and there are cases where increased anisocytosis is the main or even only abnormal feature, a serious error will be committed. Increased anisocytosis means a broader total spectrum, the distance between the outer edge of the red ring and the innermost violet ring being larger than normal. It is one of the advantages of the cell tester that such an increase in anisocytosis can be read at a glance, through the direct comparison with normal blood (Fig. 3).

TECHNIQUE OF READING RESULTS AND MEASURING

The essence of good work with blood films for any purpose is even distribution of cellular elements over a glass surface. Measurements, whether by microscopic methods or by diffraction, can only be reliable if the red cells lie separate over a large area. Any diffraction method needs a circular area of this kind of at least a quarter of an inch in diameter. If there are many cells lying partly on top of one another, one does not measure the diameter of single cells, and the results will be vitiated. A properly made blood film, made by pushing the polished narrow edge of a microscope slide along the surface of another microscope slide, with a drop of blood inside the angle made by the two slides, shows a silky glossy area at the end of the film. The speed of the movement and the size of the angle determine the quality of the film; practice is essential to acquire the art. It is only the silky area at the end of the film that can be used for diffraction measurements (Fig. 5). A simple way of checking on the suitability of a blood film is to look through its thin silky end at a distant lamp. One should then see a number of bright rainbows round the lamp. It is only with pronounced poikilocytosis that the rainbows are not so bright.

The film is moved about on the stage of the apparatus over the little hole which admits the light until one has found the spot which produces the brightest and clearest colors. This is a matter of experience.

On the screen of a properly designed apparatus for the diffraction measurement of red blood cells, such as the blood cell tester, one sees two sets of con-

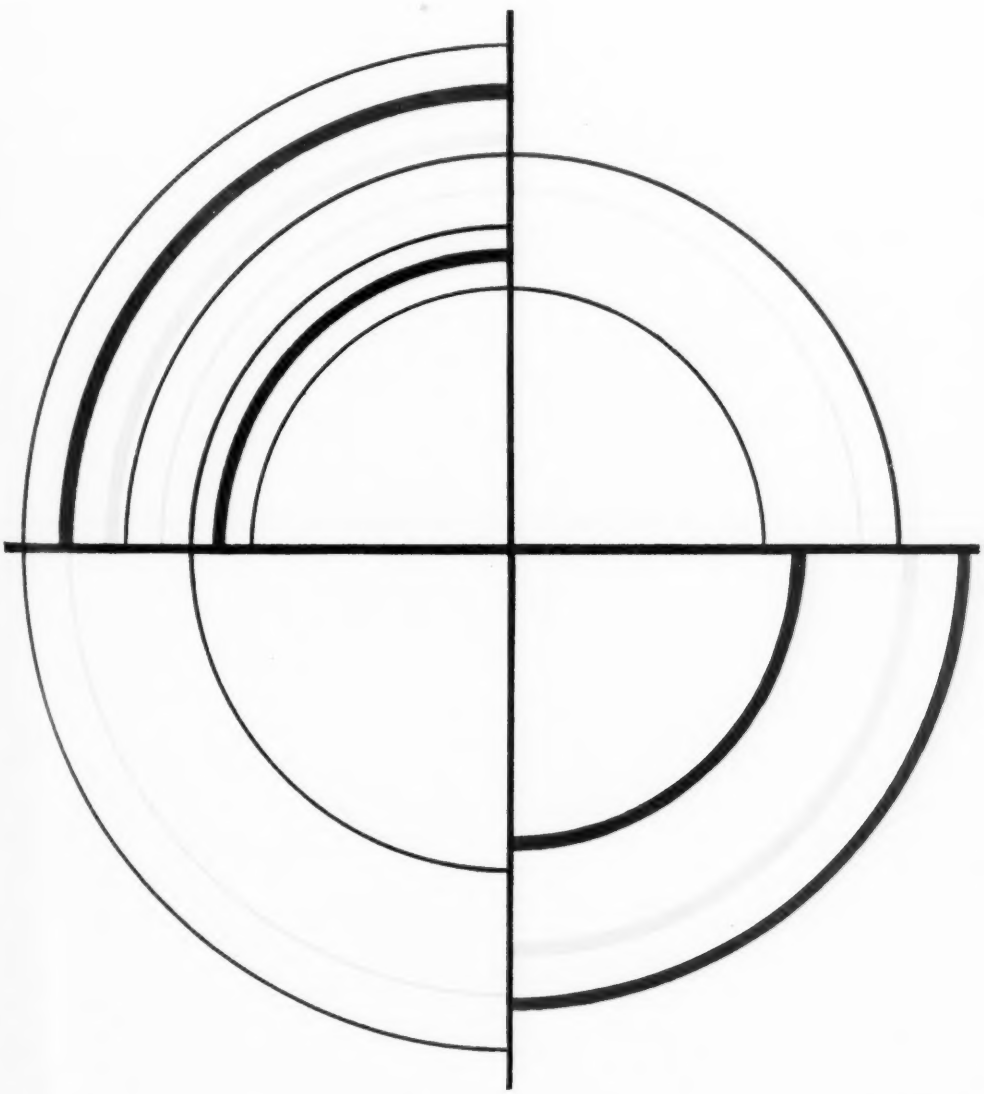


Fig. 4.—Superposition of spectral rings produced by the varying diameters of the red blood cells in a normal film.

centric colored circles arranged in rainbow fashion, as in Fig. 3. Starting from the center, where a bright patch of white light is covered by a small black shield, and traveling to the periphery, one first goes through a reddish-brown circle. Then follows the violet of the spectrum that is used for measurement and comparison. The further colors of the rainbow appear in orderly fashion, blue, green, yellow, orange, and red. The red circle is surrounded by a green circle, which belongs to the next spectrum and which for reasons into which I need not go now is somewhat confused. After this green there may be another red circle, followed again by green, and so on. For purposes of measurement and comparison one need only to take notice of the complete spectrum ranging from the innermost violet to the outer edge of red. In Fig. 3 it is at once obvious that the total spectrum on the right-hand side is a good deal wider than the one on the left. The one on the left is produced by a normal blood film and serves as a standard. There are minor individual variations in what is usually regarded as normal blood, but as a rule a properly prepared blood film made from a healthy person with a hemoglobin content of over 15 Gm. per 100 ml. will serve.

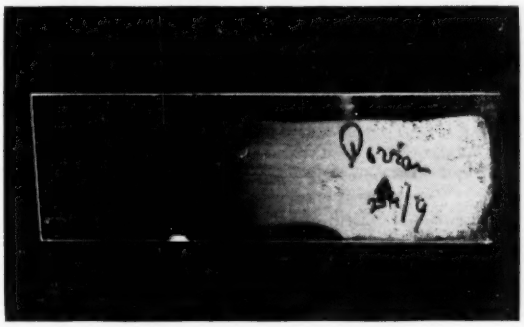


Fig. 5.—Properly made blood film, the thin silky area at the end to be used.

Such a standard blood film, unfixed and unstained, will remain serviceable for many months. In Fig. 3 it is further noticed that the yellow circle on the right side, produced by the blood under examination, is of about the same width as that of the standard blood on the left. The yellow circle being the expression of the mean diameter of the red blood cells, it becomes obvious that the mean diameter of the red blood cells under examination can be regarded as normal. If absolute values for this mean diameter are desired, a pair of compasses is used, placing the sharp point of one of the legs on the yellow circle where it touches the black line dividing the two sets of colored circles, and then the sharp point of the other leg across the center on the opposite section of the yellow circle. The distance between the two ends of the compasses is then measured on a millimeter rule and the value found converted into microns by means of the conversion table (Table III) which is based on the formula previously given. For the mean diameter one uses the column in Table III marked Yellow. If, for example, the value found in millimeters is 54, this corresponds in the conversion table to a value of 7.5 microns for the mean diameter. With practice this procedure can be performed with an accuracy of 1 mm., which corresponds to 0.1 or 0.2 microns.

The extreme edge of the red circle is the expression of the diameter of the smallest cells present in appreciable numbers in the blood film. In Fig. 3 the spectrum on the right-hand side shows that this edge extends much farther outward than with the normal blood on the left-hand side. This means that there is increased anisocytosis in the blood under examination, and this is confirmed by the innermost violet circle which is the expression of the diameter of the largest cells present in appreciable numbers and which is situated much farther inwards than in the case of the normal blood. For absolute measurements one proceeds as follows. For the extreme edge of the red circle place the sharp points of the compasses where the green circle which surrounds the extreme edge of the red one just begins, so as to make sure that the extreme edge of the red circle where the red color is faint is really taken into account. For the innermost violet, measure as near the brownish-red inner circle as possible. The millimeter values found are transferred to a millimeter rule and converted into microns by means of the conversion table (Table III), using the appropriate columns for Red and Violet. Here, too, the error should not exceed 1 mm., which corresponds to an accuracy of 0.1 or 0.2 micron. In extreme cases the error becomes larger, but also proportionally less important.

TABLE III. CONVERSION TABLE

RING IN MILLIMETERS	CELL DIAMETERS IN MICRONS			RING IN MILLIMETERS	CELL DIAMETERS IN MICRONS		
	YELLOW	VIOLET	RED		YELLOW	VIOLET	RED
25	16.5	11.7	18.3	54	7.5	5.3	8.3
26	15.8	11.2	17.6	55	7.3	5.2	8.1
27	15.2	10.8	16.9	56	7.2	5.1	8.0
28	14.6	10.4	16.3	57	7.1	5.0	7.9
29	14.1	10.0	15.7	58	6.9	4.9	7.7
30	13.6	9.7	15.2	59	6.8	4.8	7.6
31	13.2	9.3	14.7	60	6.7	4.7	7.5
32	12.8	9.0	14.2	61	6.6	4.7	7.3
33	12.3	8.7	13.8	62	6.5	4.6	7.2
34	12.0	8.5	13.3	63	6.4	4.5	7.1
35	11.6	8.2	12.9	64	6.3	4.4	7.0
36	11.3	8.0	12.6	65	6.2	4.4	6.9
37	11.0	7.8	12.2	66	6.1	4.3	6.8
38	10.7	7.6	11.9	67	6.0	4.2	6.7
39	10.4	7.4	11.6	68	5.9	4.2	6.6
40	10.1	7.2	11.3	69	5.8	4.1	6.5
41	9.9	7.0	11.0	70	5.7	4.1	6.4
42	9.6	6.8	10.7	71	5.6	4.0	6.3
43	9.4	6.7	10.5	72	5.6	3.9	6.2
44	9.2	6.5	10.2	73	5.5	3.9	6.1
45	9.0	6.4	10.0	74	5.4	3.8	6.0
46	8.8	6.2	9.8	75	5.3	3.8	5.9
47	8.6	6.1	9.6	76	5.3	3.7	5.9
48	8.4	6.0	9.4	77	5.2	3.7	5.8
49	8.2	5.8	9.2	78	5.1	3.6	5.7
50	8.1	5.7	9.0	79	5.1	3.6	5.6
51	7.9	5.6	8.8	80	5.0	3.5	5.6
52	7.7	5.5	8.6	81	4.9	3.5	5.5
53	7.6	5.4	8.5				

Conversion table to be used in connection with Pijper's Blood Cell Tester, or any other diffraction apparatus which uses a lens of 20 cm. focal distance. The diameters of the colored rings of the brightest and complete spectrum are given in millimeters, and from these the cell diameters can be found in the table. Thus, a yellow ring of 54 mm. would mean a mean cell diameter of 7.5 microns; a red ring of 64 mm. would mean that the smallest cells present in appreciable numbers would measure 7.0 microns; and a violet ring of 36 mm. would mean that the largest cells present in appreciable numbers would measure 8.0 microns.

The directions for reading and measuring and the conversion table (Table III) given here are based on theoretical considerations, amplified by long experience and supported by actual comparison of results obtained by direct microscopic methods and the diffraction method.

CLINICAL APPLICATIONS OF DIFFRACTION METHOD AND COMPARISON WITH MICROSCOPIC MEASUREMENTS

A comparison of the results of direct microscopic measurements with the results of the diffraction method applied to a small number of cases has been published.[32] It was found that the results corresponded quite well. Similar work, but in less detail, has been undertaken with essentially the same results by Bernstein,[3] Smith,[45] Knoche,[16] and especially Haden.[11, 12]

I have now collected a large number of cases with two sets of measurements undertaken on the same blood film, one by the diffraction method as detailed in this paper and one by fixing and staining the cells and then measuring them with the filar micrometer eyepiece of Bausch and Lomb. With the microscopic method 500 cells were measured in every case, taking the cells as they came, avoiding any temptation toward selection. The results of the microscopic measurements were then plotted in the form of a Price-Jones curve. These are given in Figs. 6 to 48, and underneath each curve the results of the diffraction measurements are given by three crosses. The middle cross indicates the mean diameter, and the other two indicate the diameters of the largest and smallest cells present in appreciable numbers. From the position of the crosses one can read off the degree and kind of anisocytosis present.

It cannot be expected that complete correspondence will be reached in all cases. The shape of a Price-Jones curve is the expression not only of variation in cell diameter, but also of variation in error committed in the computation of cell diameter. In addition, there is the "personal equation." It is my experience that no two technical assistants will return the same curve from one and the same blood film. The personal equation counts for much less with the diffraction method if it is performed as described herein, because there always is the direct comparison with normal blood. It follows from the nature of the diffraction method that the very smallest and the very largest cells are lost sight of, their number being too small to produce an appreciable amount of color. On the other hand, the diffraction method deals with at least a million cells, as against a few hundred dealt with by microscopic measurements, and with the diffraction method "selection" is excluded.

The diffraction method requires experience and good color vision, especially where through extensive anisocytosis the colors are spread over a larger area and become somewhat thin. Microscopic measurements, however, demand similar qualities. Checking results is easy with the diffraction method because it works so quickly, but it is hardly feasible with microscopic measurements without wasting an enormous amount of time. Marked poikilocytosis naturally causes watery spectra, somewhat difficult to measure, but then there always is the direct comparison with normal blood. Nobody who has tried it can be very

satisfied with microscopic measurements in such a case. A case of pronounced pernicious anemia may through poikilocytosis give an initial blood film with a very small spectrum and poorly defined colors, but this as such already represents useful information. It becomes even more useful if after a week's treatment, the colors, in films prepared then, become more defined; if the two films are then placed side by side in the apparatus, even minor improvements in poikilocytosis can be read at a glance. One can repeatedly collect films from such a patient and directly compare them in succession with one another in the apparatus and so get a complete picture of the progress of the case.

Figs. 6 to 48 refer to a selection of cases in which red cell measurements were undertaken on blood films, both by the diffraction method and by microscopic measurement.

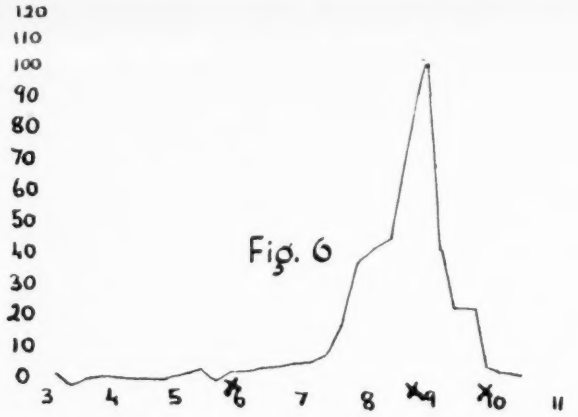
Figs. 49 and 50 illustrate how cases can be followed by means of the diffraction method. Each horizontal line gives the cell diameters of a patient on a particular day, the three crosses representing, respectively, the mean diameter (middle cross) and the smallest and largest cells (the other two crosses). The values can be read off in microns. The first 8 lines marked PA 1 refer to a patient with pernicious anemia and give the cell diameters in consecutive weeks, the first line indicating the position before treatment started, the second line after one week's treatment, and so on, until after seven weeks more or less normal conditions are reached. The difference after one week was already striking, and the improvement continued every week. The larger cells showed a more rapid return to normal than did the mean diameter, and the mean diameter again changed more quickly and markedly than the smallest cells.

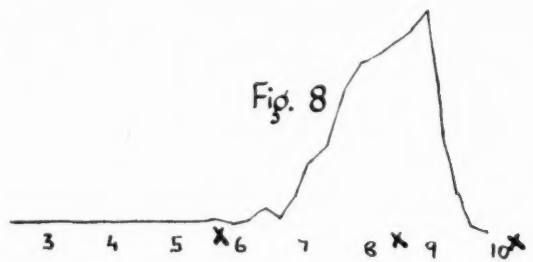
The following five lines, marked PA 2, in similar fashion refer to another patient with pernicious anemia, except that the first interval here was only four days, the other intervals again being a full week. Obvious improvement took place within the short time of four days' treatment, which was continued during the further weeks. Examinations stopped before full restoration was reached.

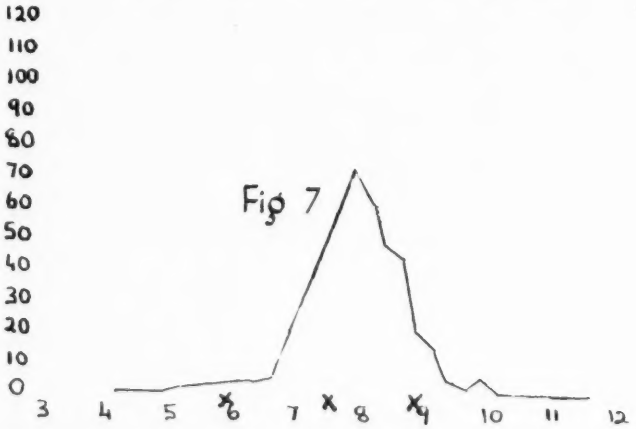
The next seven lines, marked PA 3, refer to a patient with pernicious anemia who was examined every week. Improvement was quite definite after one week, and more so after a fortnight, but then the patient neglected treatment for two weeks; this is reflected in the cell diameters, the cells increasing again in size, with anisocytosis becoming more marked. Treatment was instituted again, and improvement in cell diameters was again achieved, but the patient responded poorly and progress was not satisfactory, as shown by the cell diameters.

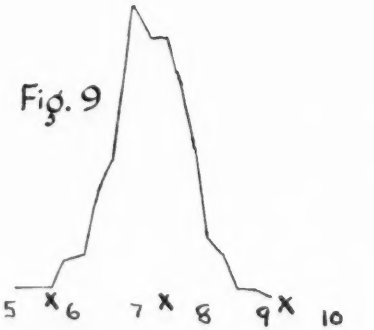
The lines and crosses marked PA 4, PA 5, PA 6, PA 7, PA 8, and PA 9 all refer to patients with pernicious anemia who were not followed for comparably long periods; the intervals between the separate individual observations varied between a fortnight and three weeks. Case PA 8 neglected treatment and this becomes manifest in the cell diameters.

These further observations confirm that at every control examination during progress it is always the largest cells that show the biggest relative improve-









Figs. 6-48.—Price-Jones curves of various blood films, with the results of diffraction measurements indicated by crosses underneath.

Fig. 6.—Old case of pernicious anemia; treatment recently neglected. Hemoglobin, 11.6 Gm.; red cells, 4.6 million. Correspondence between two methods of measurement quite good. Just as in several of the following figures, it is noticeable that the diffraction method missed the very small and the very large cells which were present in very small numbers anyhow.

Fig. 7.—Same case as in Fig. 6, but now had had good treatment for four months. Hemoglobin, 14.5 Gm.; red cells, 5.2 million. Correspondence between two methods not so good, but "shift to left" and decrease in anisocytosis obvious by both methods.

Fig. 8.—Untreated case of pernicious anemia. Hemoglobin, 8.2 Gm.; red cells, 2.9 million. Correspondence quite good.

Fig. 9.—Same case as in Fig. 8; had now had treatment for two months. Hemoglobin, 13.4 Gm.; red cells, 5.5 million. Correspondence good; anisocytosis more pronounced with diffraction method than with microscopic method.

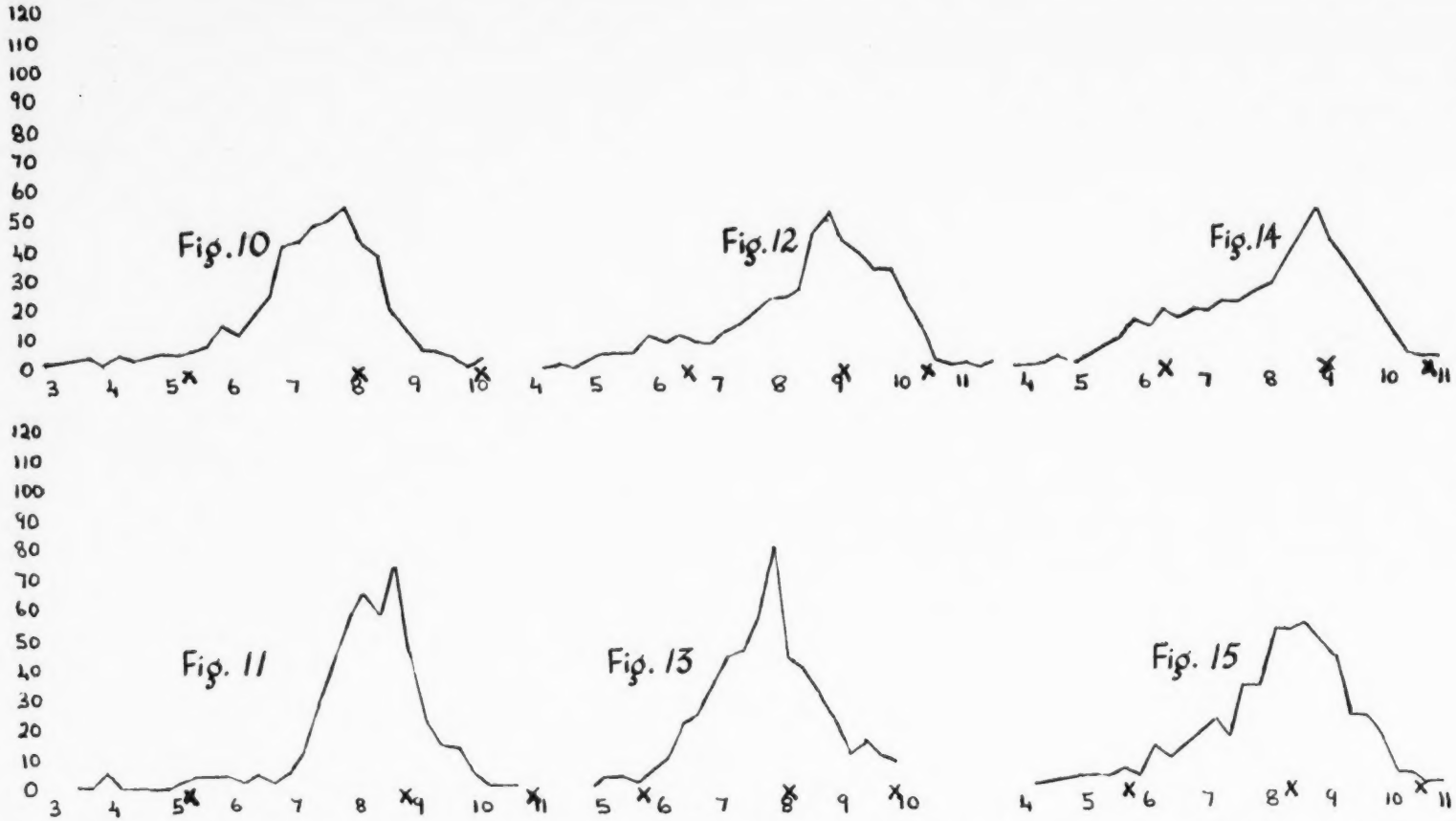


Fig. 10.—An Indian man, with carcinoma of liver. Hemoglobin, 4.4 Gm.; red cells, 1.3 million. Note "shift to right" and very marked anisocytosis brought out by both methods.

Fig. 11.—Pernicious anemia, untreated. Hemoglobin, 6.1 Gm.; red cells, 1.6 million. Both methods showed very similar conditions of red cells; the diffraction method showed more anisocytosis.

Fig. 12.—Pernicious anemia, untreated. Hemoglobin, 4.4 Gm.; red cells, 2.0 million. Correspondence quite good, except for some more smaller cells showing up by microscopic method.

Fig. 13.—Same case as in Fig. 12, but now had had several treatments for several weeks. Hemoglobin, 9.9 Gm.; red cells, 3.7 million. Note "shift to left," but cells still remained large and anisocytosis very marked.

Fig. 14.—Pernicious anemia. Hemoglobin, 6.7 Gm.; red cells, 2.8 million. Correspondence good except for some smaller cells, which had got lost with the diffraction method. In essence, however, both methods agree quite well.

Fig. 15.—Same case as in Fig. 14, but now had had treatment for two weeks. Hemoglobin, 9.7 Gm.; red cells, 3.2 million. Note slight but identical "shift to left" coming to light by both methods. Cells still very large and anisocytosis very marked.

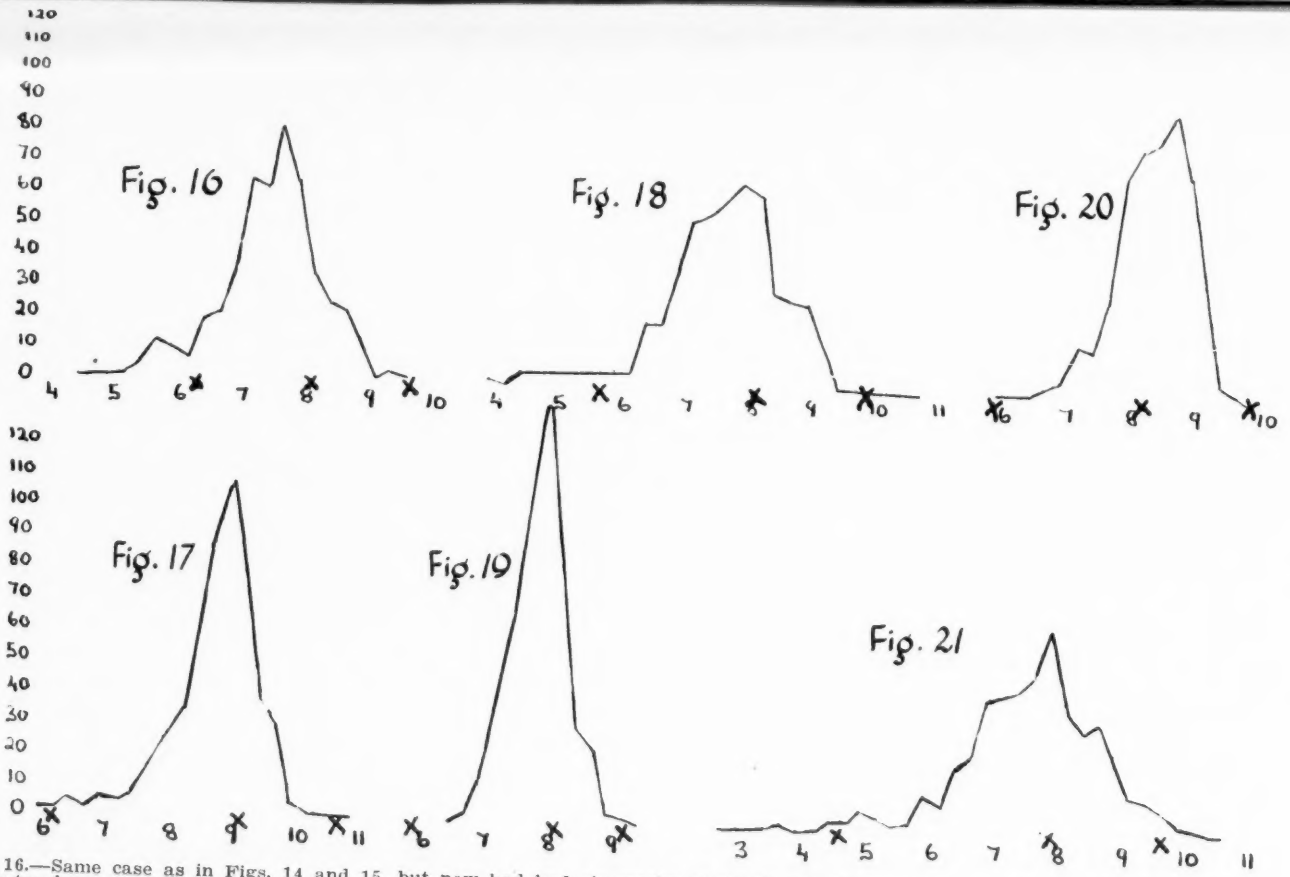


Fig. 16.—Same case as in Figs. 14 and 15, but now had had six weeks of treatment. Hemoglobin, 14 Gm.; red cells, 5.1 million. Cells still rather too large and too much anisocytosis, both abnormalities appearing more pronounced with diffraction method.

Fig. 17.—Pernicious anemia; had had irregular treatment. Hemoglobin, 12.6 Gm.; red cells, 4.7 million. Cells still much too large, anisocytosis more than normal, both changes more obvious with diffraction method.

Fig. 18.—Insufficiently treated pernicious anemia. Hemoglobin, 5.1 Gm.; red cells, 2.6 million. Note marked anisocytosis and large cells, as shown by both methods.

Fig. 19.—Subacute combined degeneration of spinal cord. Hemoglobin, 13.9 Gm.; red cells, 5.7 million. Note large cells with hardly more anisocytosis than normal. This is the kind of case that is quickly diagnosed by diffraction method.

Fig. 20.—Obscure jaundice in child. Hemoglobin, 14.3 Gm.; red cells, 5.3 million. Cells large, with marked anisocytosis, rather similar picture by both methods.

Fig. 21.—Pernicious anemia. Hemoglobin, 5.9 Gm.; red cells, 2.0 million. Correspondence very good.

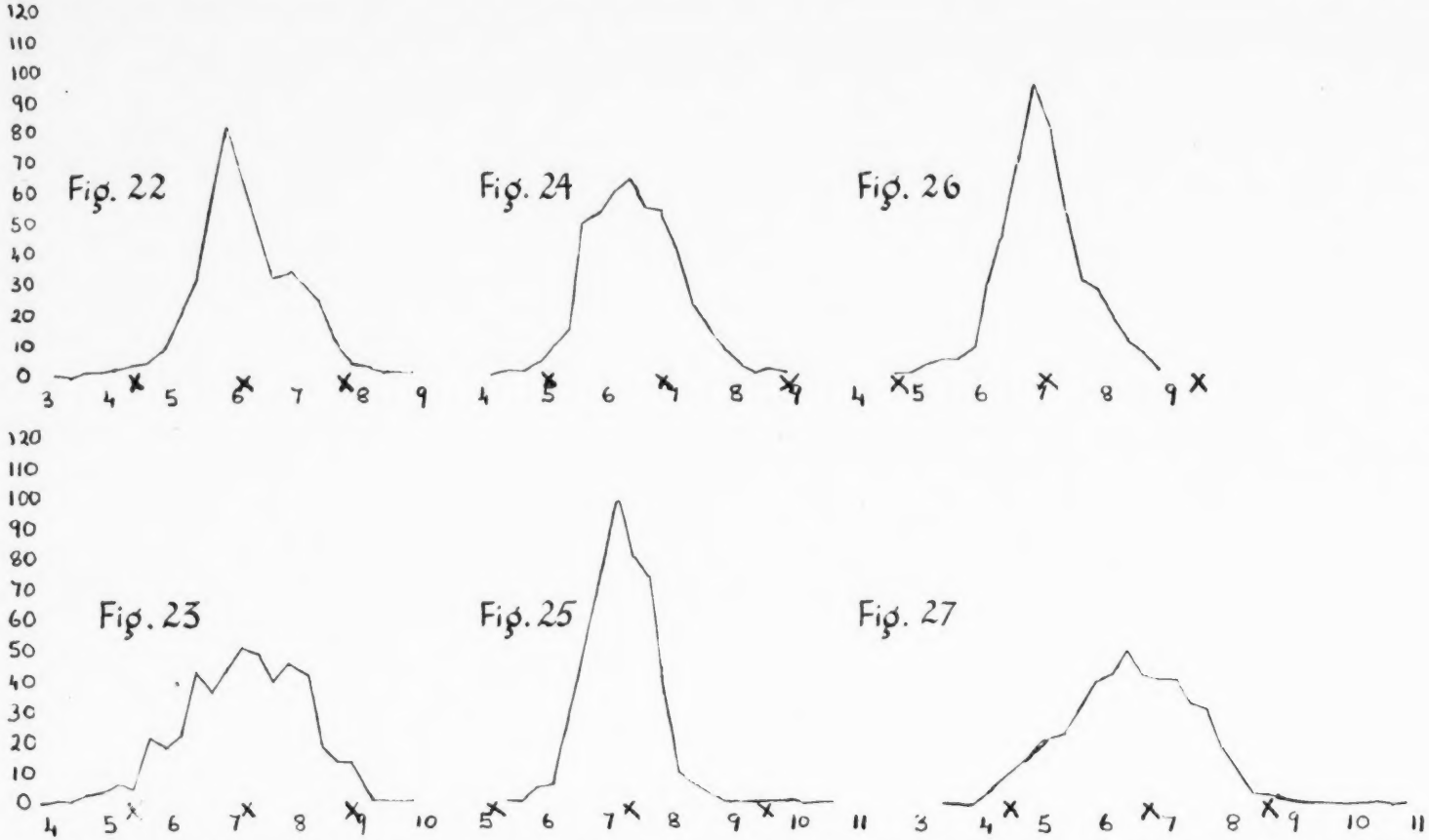


Fig. 22.—Familial hemolytic jaundice. Hemoglobin, 6.9 Gm.; red cells, 3.8 million. Fragility of red cells: hemolysis started at 0.5 per cent, was complete at 0.38 per cent. Spleen taken out next day. Note especially smallness of cells, which suggested diagnosis in diffraction apparatus.

Fig. 23.—Same case as in Fig. 22: spleen taken out two weeks before. Hemoglobin, 7.9 Gm.; red cells, 5.7 million. All cells shifted about a whole micron to the right, but anisocytosis much the same.

Fig. 24.—Carcinoma of colon. Hemoglobin, 4.4 Gm.; red cells, 3.6 million. Cells rather small, anisocytosis increased, picture was difficult to interpret, but excluded pernicious anemia and urged examiner to look for other causes.

Fig. 25.—Prolonged bleedings from hemorrhoids. Hemoglobin, 8.7 Gm.; red cells, 2.9 million. Mean diameter normal, but anisocytosis marked, much more so by diffraction method than by microscopic method.

Fig. 26.—Prolonged uterine hemorrhages. Hemoglobin, 6.3 Gm.; red cells, 3.3 million. Note normal mean diameter and again marked anisocytosis.

Fig. 27.—Prolonged uterine hemorrhages. Hemoglobin, 4.8 Gm.; red cells, 2.3 million. Mean diameter somewhat small by both methods, but main feature was extreme anisocytosis, also readily visible by both methods.

Fig. 26.—Prolonged uterine hemorrhages. Hemoglobin, 6.3 Gm.; red cells, 3.3 million. Note normal mean diameter and again marked anisocytosis.

Fig. 27.—Prolonged uterine hemorrhages. Hemoglobin, 4.8 Gm.; red cells, 2.3 million. Mean diameter somewhat small by both methods, but main feature was extreme anisocytosis, also readily visible by both methods.

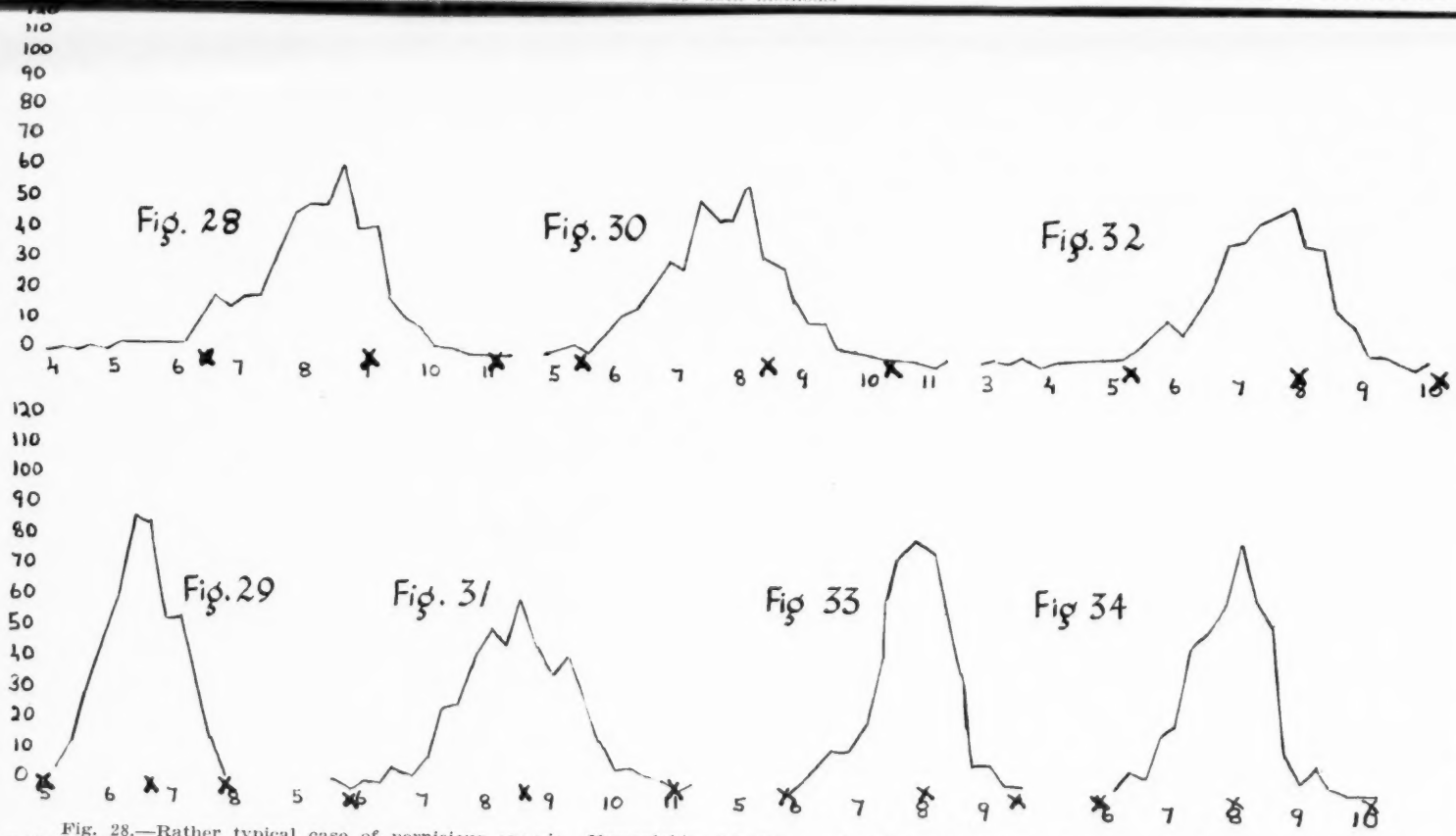


Fig. 28.—Rather typical case of pernicious anemia. Hemoglobin, 8.5 Gm.; red cells, 3.0 million. Cells appeared larger by diffraction method, but main features of both sets of data were identical.

Fig. 29.—Acholuric jaundice. Hemoglobin, 14.1 Gm.; red cells, 4.7 million. Fragility: started at 0.56 per cent, was complete at 0.40 per cent. Cells very small; anisocytosis increased.

Fig. 30.—Pernicious anemia. Hemoglobin, 8.9 Gm.; red cells, 2.3 million. Mean diameter somewhat larger by diffraction than by microscopic measurement; anisocytosis very marked and identical by both methods.

Fig. 31.—Pernicious anemia. Hemoglobin, 12.4 Gm.; red cells, 3.9 million. Cells large; excellent correspondence between the two methods.

Fig. 32.—Liver carcinoma. Hemoglobin, 6.5 Gm.; red cells, 2.1 million. Cells enlarged; correspondence quite good.

Fig. 33.—Treated pernicious anemia. Hemoglobin, 15.4 Gm.; red cells, 4.9 million. Cells still large and increased anisocytosis; identical by both methods.

Fig. 34.—Long and well-treated pernicious anemia. Cells, however, still large and increased anisocytosis; identical by both methods. Hemoglobin, 13.4 Gm.; red cells, 4.2 million.

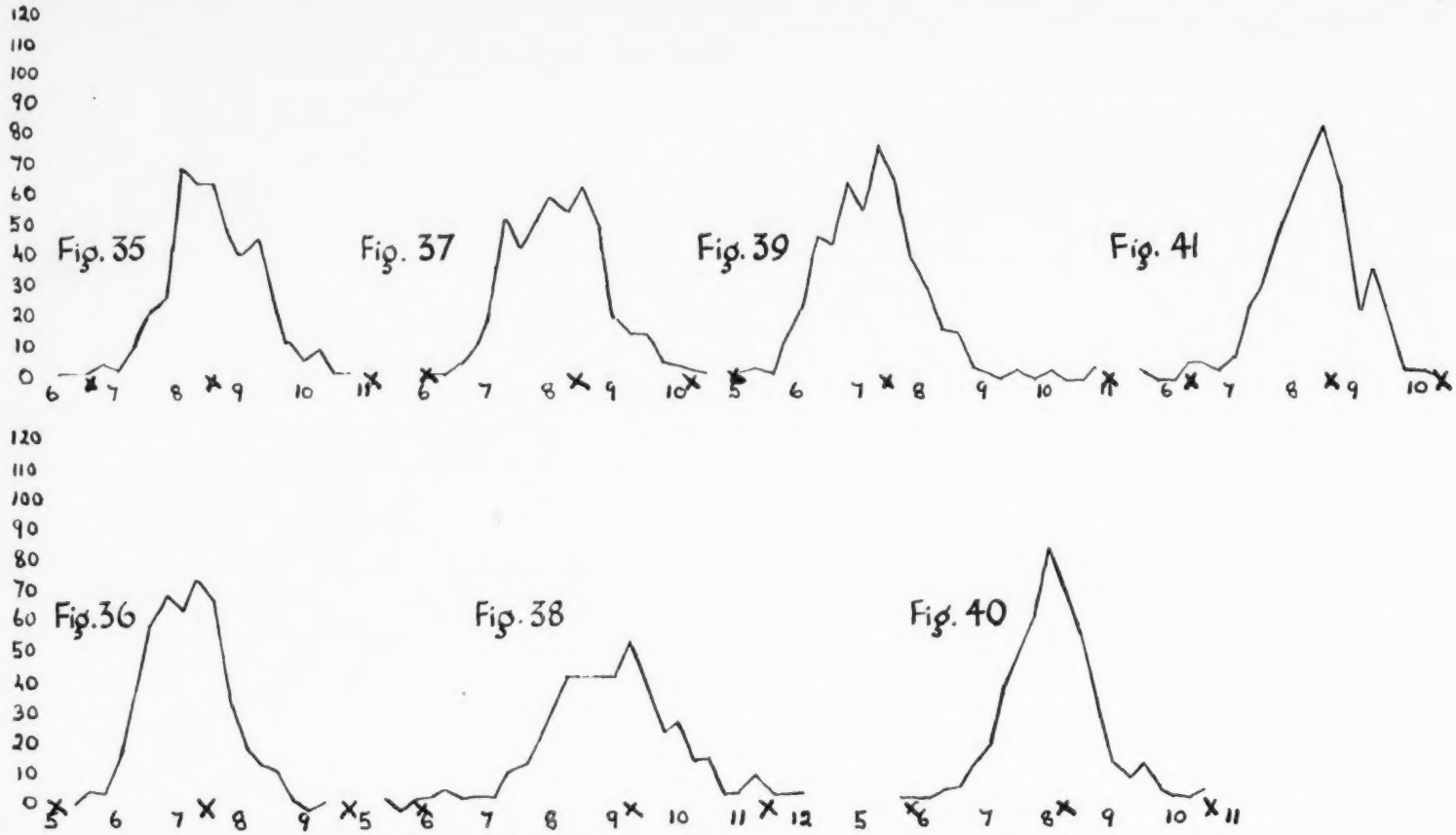


Fig. 35.—Pernicious anemia. Hemoglobin, 12.7 Gm.; red cells, 3.5 million. Diameters large, with increased anisocytosis; correspondence quite good.

Fig. 36.—Anemia from uterine hemorrhages. Hemoglobin, 9.8 Gm.; red cells, 3.1 million. Mean diameter normal by both methods; anisocytosis increased, more pronounced with diffraction method.

Fig. 37.—Pernicious anemia. Hemoglobin, 13.1 Gm.; red cells, 3.1 million. Cells enlarged, anisocytosis increased, correspondence quite good.

Fig. 38.—Pernicious anemia. Hemoglobin, 8.2 Gm.; red cells, 2.2 million. Cells large, and the anisocytosis was extreme; very well shown by both methods.

Fig. 39.—Anemia through prolonged mental illness, neglect, and starvation. Hemoglobin, 3.8 Gm.; red cells, 2.1 million. Mean diameter normal; chief abnormality was anisocytosis which was increased, more so by the diffraction method than by microscopic measurement.

Fig. 40.—Pernicious anemias; marked nervous symptoms. Hemoglobin, 11.1 Gm.; red cells, 2.6 million. Mean diameter large and same by both methods; anisocytosis increased, more marked by diffraction method than by microscopic measurements.

normal; chief abnormality was anisocytosis which was increased, more so by the diffraction [illegible]

Fig. 40.—Pernicious anemias; marked nervous symptoms. Hemoglobin, 11.1 Gm.; red cells, 2.6 million. Mean diameter large and same by both methods; anisocytosis increased, more marked by diffraction method than by microscopic measurements.

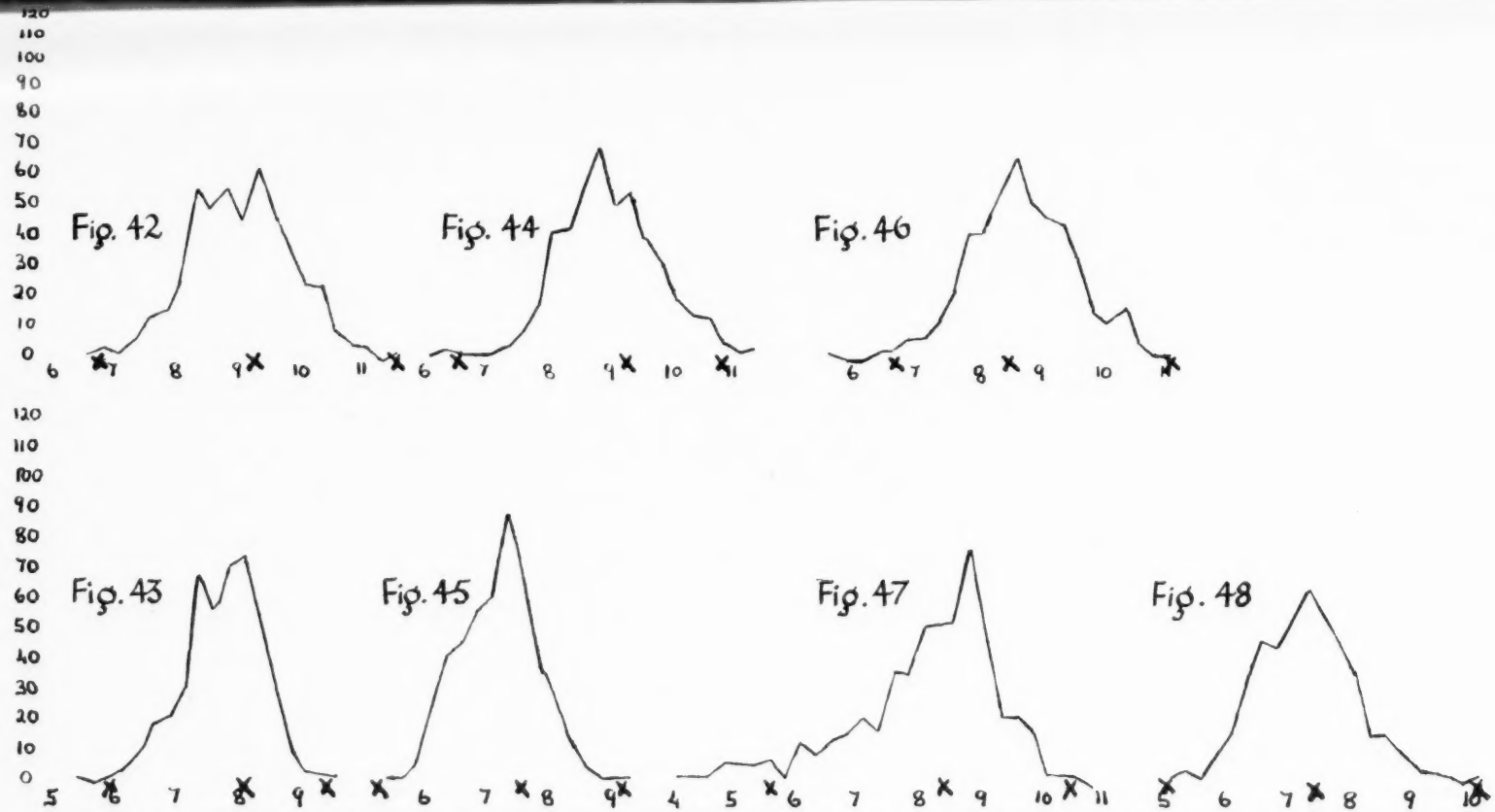


Fig. 41.—Obscure liver disease in native woman. Hemoglobin, 13.9 Gm.; red cells, 3.3 million. Cells enlarged, anisocytosis increased, good correspondence.

Fig. 42.—Pernicious anemia. Hemoglobin, 11.2 Gm.; red cells, 3.2 million. Cells very large, anisocytosis increased, very good correspondence.

Fig. 43.—Pernicious anemia. Hemoglobin, 13.1 Gm.; red cells, 3.9 million, marked nervous symptoms. Cells rather large, anisocytosis somewhat increased, good correspondence.

Fig. 44.—Pernicious anemia. Hemoglobin, 7.0 Gm.; red cells, 2.5 million. Cells very large and extensive anisocytosis, rather more marked with diffraction method.

Fig. 45.—Anemia in native woman with syphilis. Mean diameter, normal, anisocytosis increased, rather more pronounced with diffraction method. Hemoglobin, 8.5 Gm.; red cells, 3.1 million.

Fig. 46.—Pernicious anemia. Hemoglobin, 10.9 Gm.; red cells, 3.2 million. Cells enlarged, anisocytosis increased, good correspondence.

Fig. 47.—Pernicious anemia. Hemoglobin, 8.0 Gm.; red cells, 2.7 million. Cells enlarged, but striking feature was very much increased anisocytosis, obvious by both methods.

Fig. 48.—Anemia through malignant growth. Hemoglobin, 10.2 Gm.; red cells, 3.5 million. Mean diameter normal, anisocytosis much increased, good correspondence.

ment; then come the cells of mean diameter and, last, the smallest cells. Another important feature is that the diffraction method is sensitive enough to show definite improvement in a week or less when proper treatment is instituted and may therefore take the place of the more complicated counting of reticulocytes.

The case marked FAJ 1 suffered from familial acholuric jaundice and was examined twice, the first time for diagnostic purposes, and then again a fortnight after splenectomy. Here, too, the improvement is brought out very clearly by the diffraction method.

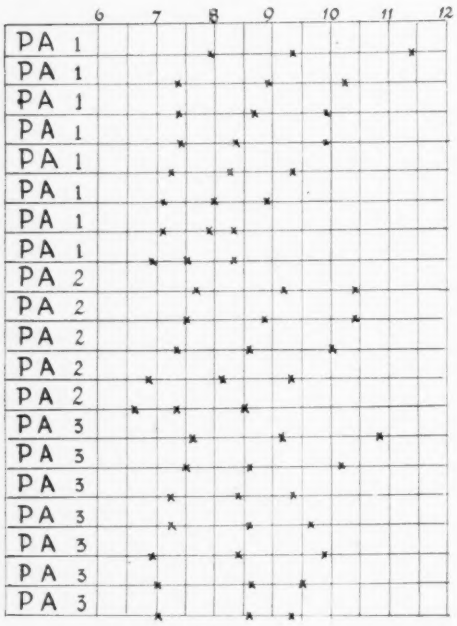


Fig. 49.

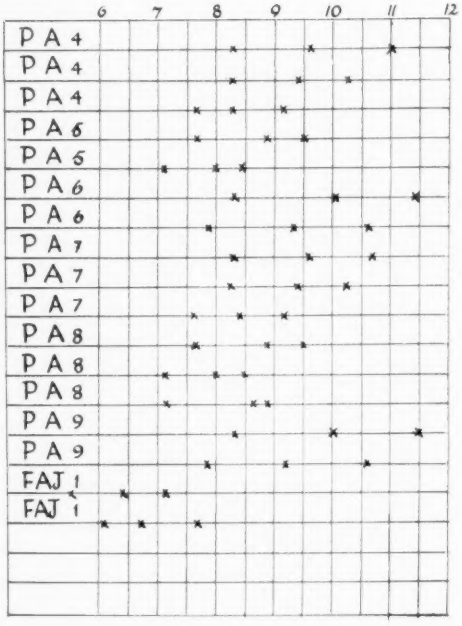


Fig. 50.

Figs. 49 and 50.—Illustrate the results of diffraction measurements in the follow-up of patients.

SUMMARY

A historical introduction shows how blood films came to be used as diffraction gratings for the measurement of red cells, and how the technique was further developed, resulting in Pijper's Blood Cell Tester. This is a very simple apparatus which can easily be constructed from simple materials. Its leading feature is that it permits the direct comparison on one and the same screen of the diffraction spectra produced by two different blood films. Since the diffraction spectra are the expression of the red cell diameters, it follows that one can directly compare the cell diameters of standard normal blood with those of a patient, or of two blood films from the same patient, taken at different times. This arrangement on occasion does away with the necessity for actual measurement; a glance at the screen may be sufficient to decide what sort of anemia one is dealing with or whether the patient is making progress. Improvement in cell diameters after a few days of treatment can readily be observed by direct comparison of two blood films in the apparatus.

The advantages of this "duplication" technique and apparatus are compared with the various instruments suggested by other authors, which, although based on the same original idea, do not always do full justice to the possibilities of the diffraction method.

The main purpose of the paper is to show that with the diffraction method in the form finally developed, it is possible not only to determine the mean diameter, but also the degree and quality of anisocytosis present in a blood film. These determinations do not take more than a few minutes, which means a great saving in time as compared with the making of a Price-Jones curve. This diffraction method gives, in a fraction of the time, practically the same information as the laborious microscopic measurements of Price-Jones. It also takes into account a much larger number of cells. Its speed makes it readily applicable to any patient.

For differential diagnosis the importance of determining red cell diameters is admittedly great. The diffraction method in its proper form as described herein does this directly and very reliably. The indirect methods aiming at similar information by determining hemoglobin index or mean cell volume cannot be as reliable because it is necessary to divide two doubtful values through one another, and this may lead to gross distortion of actual conditions, as shown in this paper. Also, these indirect methods give mean values only, not degree and quality of anisocytosis.

The opportunity is taken of pointing out that blood films must be made with adequate skill and care to be of use in diffraction measurements. It seems that disappointments with the diffraction method in many instances are due to indifference in this respect.

In this paper are discussed the theoretical foundations of the diffraction method, and the technique of making accurate measurements is given in detail. A conversion table (Table III) is included which translates the diameters of the colored rings into microns, both for mean diameter and degree and quality of anisocytosis. This table is suitable for any apparatus working with a lens of 20 cm. focal distance.

A series of cases is described in which both microscopic measurements and complete diffraction measurements were performed, the identical blood films being used for both methods. It is seen that the results correspond quite well.

Finally, the usefulness of the diffraction method for following cases under treatment is illustrated.

PRACTICAL INSTRUCTIONS FOR BUILDING A DIFFRACTION APPARATUS

A suitable apparatus embodying the principles of Fig. 2 can easily be built, following the lines of the Pijper's Blood Cell Tester, of which an illustration is given in Todd and Sanford[46] and in other textbooks. One needs a wooden box, at the bottom of which is placed a small electric lamp, providing a narrow light source. A small circular diaphragm on top of the lamp produces a parallel beam of light, which reaches the horizontal stage of the apparatus, in which there are two small holes. (It would be a refinement to use a collector lens to provide a parallel beam.) In the Zeiss apparatus, contrary to the diagram, the beam strikes a simple lens, underneath the stage, before it reaches the blood films on the stage. This is the only lens which is essential. It is a simple lens of 20 cm. focal distance; a spectacle lens of

5 diopters is good enough. The horizontal stage enables one to move the blood films until the best spot is found. The vertical partition which separates the two diffraction patterns should be made of a thin piece of metal. The receiving screen forming the top of the apparatus should be of fine grain ground glass; various kinds should be tried to get best results.

I wish to thank two of my technical assistants, Mrs. J. van Sighem and Miss J. Todd, for great care and patience exercised in the making of Price-Jones curves.

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THE ROLE OF PHAGOCYTOSIS IN RESISTANCE, AS RELATED TO AGE OF GRANULOCYTES FOLLOWING PRIMARY AND REINFECTION STUDIES WITH HEMOLYTIC STREPTOCOCCI IN MACACUS RHESUS

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IN THE studies previously reported from this laboratory[1-5] which attempted to analyze the relative roles of cellular and humoral immune factors in monkeys subjected to respiratory infections with the hemolytic streptococcus, Group C, not only the quantitative response of the neutrophilic granulocytes, but also the phagocytic activity (opsonocytophagic index of Huddleson[6]) in terms of relative cell maturity were investigated. To an initial intranasal infection with the streptococcus, there was invariably an immediate response marked by an effective granulocytic leucocytosis. A later reinfection with the same organism quite as regularly *failed* to elicit any appreciable disturbance in the quantitative equilibrium of the circulating white blood cells. Yet there was no apparent change in the effective resistance of the animals. It seemed desirable, therefore, to analyze the opsonic index under these two conditions, and at the same time to correlate the Arneth-Schilling index of maturity of the granulocytes to determine the optimum age for maximum phagocytic function under the conditions of this experimental study.

Previous studies by various workers concerning the qualitative and quantitative responses of the neutrophilic leucocytes to infection are not in agreement. Much of the discrepancy may be attributed to differences in technique and in the materials employed. Hamilton-Black[7] in 1913 observed the polymorphonuclear leucocytes during the tuberculin treatment of tuberculosis. After tuberculin he noted an increase in the number of mature cells as well as a total increase in leucocytes. Of the number of tubercle bacilli ingested (method not described) he found that neutrophiles with one nucleus engulfed 10 per cent; with two nuclei, 18 per cent; with three nuclei, 22 per cent; and with four and five nuclei, 25 per cent, respectively. Arneth[8] in 1920, in examining purulent exudates but no peripheral blood preparations, reported that only mature leucocytes (Class 2 or 3) were concerned in phagocytosis, that the very young and the senile cells did not participate. Ponder and Flinn[9] in 1926 found no difference in the phagocytic properties of cells using *Mycobacterium tuberculosis, Escherichia coli, Staphylococcus aureus,* and *Neisseria catarrhalis.* Jacobsthal[10] in 1921 used citrated preparations from acute myeloblastic leucemia, myeloid leucemia, and Hodgkin's disease with multiple skin infiltrations and high eosinophilia. After

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finding staphylococci and the anthrax bacillus unsatisfactory, he used cinnabar and *Micrococcus tetragenus*. He concluded that:

1. Neutrophilic myeloblasts and myelocytes have the ability, in vitro, to phagocytize cinnabar and bacteria.

2. Eosinophiles are phagocytic.

3. Observations on the blood of two cases of leucemia showed that adult granulocytes but not myelocytes were able to phagocytize bacteria and foreign erythrocytes.

4. Eosinophiles in cases of Hodgkin's disease readily phagocytized staphylococci, micrococci, and tubercle bacilli.

Jung[11] in 1932 noted that as the number of cells increases, the percentage of phagocytes diminishes.

EXPERIMENTAL

Materials and Methods.—Daily determinations based on a modification of Huddleson's method[6] were made in selected monkeys in order to study any variation in the phagocytic properties of the circulating granulocytes of the infected animals. Young cultures of *Streptococcus hemolyticus,* Group C, which were culturally characteristic and not resistant to phagocytosis, were employed. For each opsonic determination, cultures grown on North's chocolate gelatin agar slants for eighteen to twenty-four hours at 37° C. were used. By means of a sterile wire loop the growth was gently removed from the surface and emulsified in sterile 2 per cent sodium citrate in physiologic saline solution which served both as a diluent and anticoagulant. The suspension was then adjusted by means of a nephelometer to a standard turbidity approximately equivalent to 30 billion bacteria per milliliter. After allowing a few minutes for any clumps or particles to settle, 0.1 ml. of the suspension was introduced into sterile agglutination tubes to which an equal amount of blood freshly obtained from the marginal ear vein was added. After being well shaken, the tubes were placed in a water bath adjusted to 37° C. The tubes remained in the bath approximately one hour when they were shaken again and then incubated an additional hour at 37° C. Duplicate thick smears of the incubated blood-bacterial suspension were made on clean glass microscopic slides and dried rapidly by means of an electric fan. The slides were then placed in a 5 per cent formalin and 1 per cent acetic acid solution for four minutes in order to dissolve the red corpuscles and to fix the leucocytes, following which the slides were dried with bibulous paper and the smears stained for thirty seconds with Bordet-Gengou's toluidine blue. In determining the degree of phagocytosis, the bacteria in twenty-five polymorphonuclear cells were counted and the phagocytic power measured by the average number of bacteria engulfed by each phagocyte in contrast with the number phagocytized in the presence of blood taken previous to inoculation. So far as possible, the individual cocci were counted. Daily blood counts were made on each animal over a period of two to three weeks. The age of each granulocyte in terms of lobation of the nucleus was recorded, and the engulfed bacteria counted.

Experiment 1.—As noted in earlier studies[1-5] primary intranasal inoculation with the Group C streptococcus was followed by a sharp polymorphonucler leucocytosis but the phagocytic index was not appreciably altered. Reinoculation, however, was followed by a sharp increase in individual phagocytosis but no leucocytosis occurred. Thus, in order to evaluate quantitatively the number of bacteria phagocytized daily, the total number of bacteria engulfed was determined by multiplying the opsonic index by the total number of neutrophiles. The results obtained in the two monkeys to be discussed are representative of those obtained in a group of twenty-three animals studied.

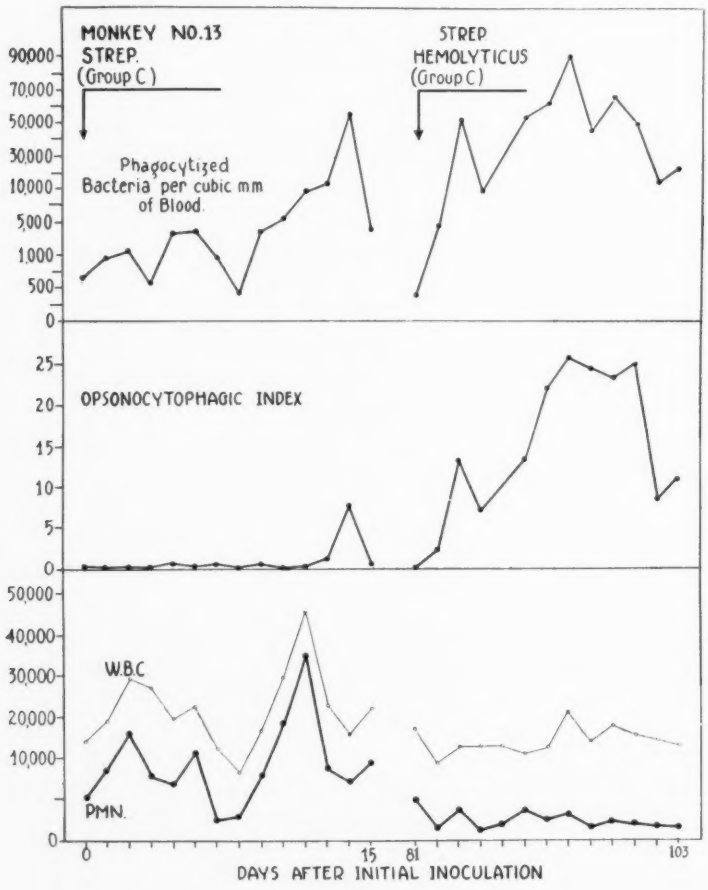

Fig. 1.

As noted in Fig. 1, Monkey 13 showed a marked polymorphonuclear leucocytosis after primary inoculation with relatively little increase in the opsonic index. However, there was a definite increase in the total number of bacteria engulfed from a preinoculation level of 672 bacteria per cubic millimeter of blood to a maximum of 55,600 bacteria on the twelfth postinoculation day, with intermediate levels varying from 456 to 13,338 bacteria per cubic millimeter. At

the time of maximum phagocytosis, the opsonic index was 8 as compared with 0.2 at the time of inoculation, while the neutrophiles totaled 7,000 as compared with 4,000. On the tenth postinoculation day the total number of bacteria engulfed was 9,660 per cubic millimeter despite a low opsonic index of 0.3 due to the neutrophilic increase (34,500 polymorphonuclears); on the eleventh day there were 13,338 bacteria phagocytized per cubic millimeter when the opsonic index was only 1.5, but the neutrophiles totaled 8,775.

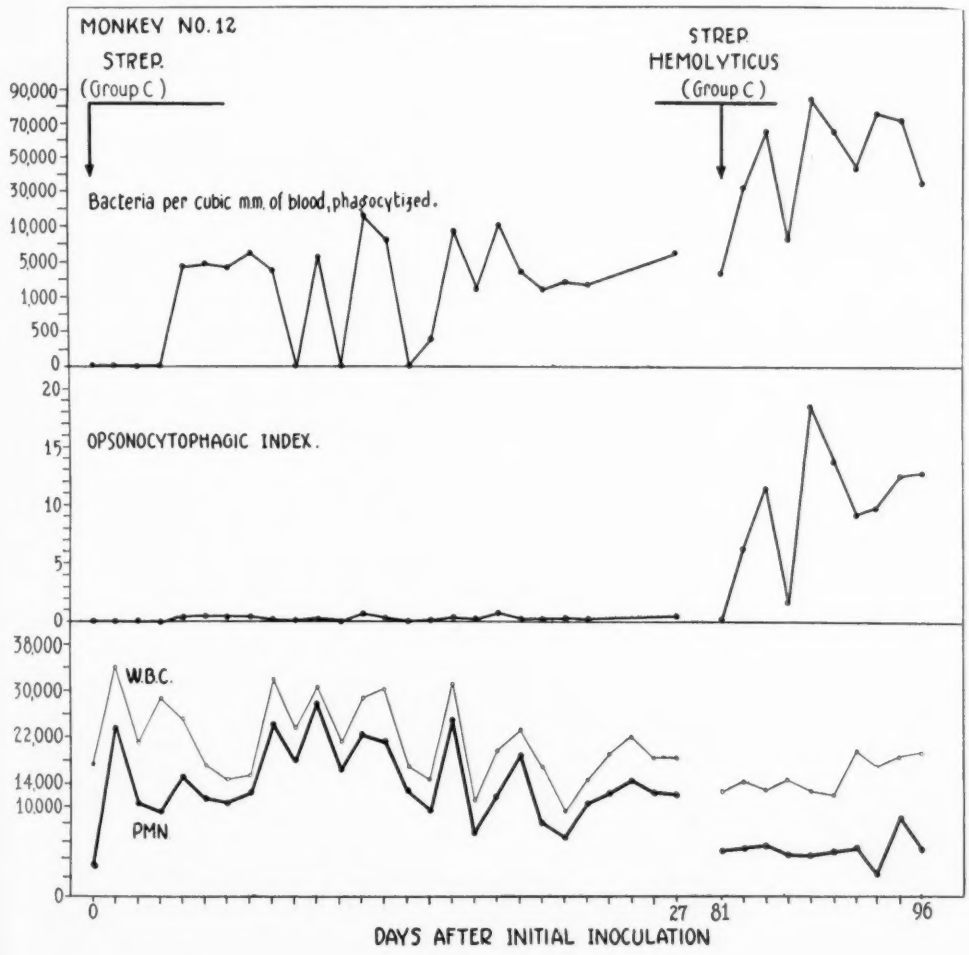

Fig. 2.

Following reinoculation, three months after primary infection, there was a consistently higher number of bacteria engulfed due primarily to the increase in individual cell phagocytosis without leucocytosis, with the highest number of bacteria engulfed being 87,000 on the seventh day after reinoculation with the streptococcus, at which time the opsonic index was 28 while total neutrophiles were only 3,310. Twenty-four hours after reinoculation with the streptococcus

the opsonic index was 2.5 and the average number of bacteria phagocytized per cubic millimeter was 50,274; then it fell to 9,855 after forty-eight hours but increased on the third day to 50,557. For the next week the average number of bacteria phagocytized per cubic millimeter varied between 45,000 and 87,000, with opsonic indices ranging between 13.5 and 25.

Thus, although it was apparent that there was increased phagocytosis per individual cell following reinoculation as compared with primary inoculation with the streptococcus, due to the greater leucocytosis following primary inoculation there was actually less difference in the degree of total phagocytosis than would be expected from evaluation of the opsonic index alone.

Similarly, as noted in Fig. 2, there was a low opsonic index with a compensatory leucocytosis following primary instillation of the streptococcus, as compared with a lack of leucocytosis but with increased individual cell phagocytosis after reinoculation. Monkey 12 is also representative of the animals showing this response. The preinoculation opsonic index of 0 in this animal showed a slight increase to only 0.3 on the fifth day after primary inoculation with the streptococcus, with resultant indices for the next three weeks varying between 0 and 0.9. However, due to the leucocytosis there was an increase in total numbers of bacteria engulfed per cubic millimeter to as high as 17,700 on the twelfth day (opsonic index, 0.8; neutrophiles, 22,137), with the average number of bacteria engulfed per cubic millimeter being about 4,000. Before reinoculation with the streptococcus three months later, the opsonic index was 0.6; neutrophiles, 5,030; and the total number of bacteria engulfed per cubic millimeter, 3,018. Within twenty-four hours the opsonic index rose to 6.1, with 33,600 bacteria engulfed per cubic millimeter, although there was no increase in neutrophiles (5,510). For the next two weeks the opsonic index averaged between 6 and 18, except for one low value of 1.8 on the third day. The total number of bacteria engulfed per cubic millimeter rose to 83,600.

Here again there was a greater degree of phagocytosis per individual cell following reinoculation with the streptococcus than after primary inoculation. A greater total bacterial phagocytosis, however, followed this primary inoculation than might have been expected from the very low opsonic index.

Experiment II.—The monkeys used in this study were animals which had been infected by intranasal inoculation with the Group C hemolytic streptococcus three to six months earlier. After reinfection the granulocytes in these animals exhibited a marked increase in phagocytic activity which had not been apparent after primary infection, without the significant initial quantitative leucocytosis.

Fig. 3 presents graphically the relative degree of phagocytic activity by the various age groups of polymorphonuclear leucocytes. Monkey 8, for example, showed a progressive phagocytic efficiency beginning with the band forms (seventeen bacteria per cell), reaching a peak of twenty-two organisms per cell in the three-lobed neutrophiles, and then showing a gradually diminishing effectiveness in the four-, five-, and six-lobed cells which handled twelve, three, and six bacteria, respectively. Monkey 6 showed a similar picture with ten, thirteen,

and eleven bacteria engulfed by the one-, two-, and three-lobed cells, respectively, while the four-, five-, six-, and seven-lobed neutrophiles each engulfed from four to seven bacteria.

The young cells in Monkey 13 likewise showed greatest phagocytic activity by the one-, two-, and three-lobed cells, while Monkey 17 showed a gradual progressive decrease in phagocytosis inversely proportional to the age of the leucocytes. The band cells in Monkeys 16, 29, and 30 had the highest phagocytic index with gradual diminution in phagocytosis as cell age increased.

In contrast, there apparently was no correlation between cell maturity and phagocytosis following primary infection. The small degree of phagocytosis observed here was apparently distributed at random among the various cells.

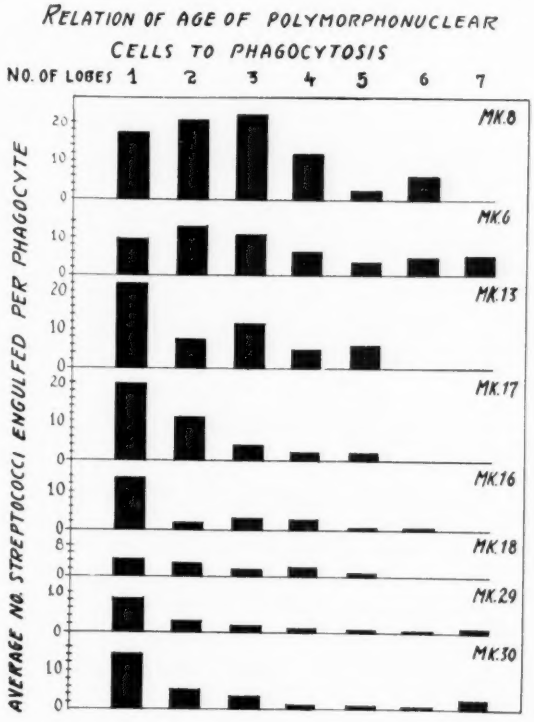

Fig. 3.

DISCUSSION AND SUMMARY

Earlier studies[1-5] from this laboratory have shown that monkeys receiving intranasal inoculations of *Streptococcus haemolyticus,* Group C, reacted with an immediate marked and clear-cut peripheral leucocytosis but without any elevation in the opsonic index as measured by in vitro tests. Reinoculation with the same agents three to six months later resulted in no appreciable leucocytosis, but there was a prompt and significant increase in phagocytosis by the individual granulocytes. If the in vitro determinations of the potential phagocytic capacities of the neutrophilic granulocytes for hemolytic streptococci multiplied

by their number per cubic millimeter in the circulating blood may be interpreted as a relative index of the cellular component of resistance to infection with these organisms, then the inverse ratio of these two phenomena, which occur in primary versus reinfection states, becomes not only interesting, but deeply significant as well. On the basis of the findings here presented, we agree with Jung,[11] that the lower the phagocytic index, the greater the number of granulocytes required to control any given infection. The absolute leucocytosis following close upon a primary infection with low opsonic index would seem to represent a compensatory response in which the resistance of the host or his cellular protection is measured by the sheer numbers of available phagocytes. When the relatively low opsonic indices are evaluated in relation to the total available polymorphonuclear leucocytes, the absolute number of bacteria phagocytized by the neutrophiles will be greater than is at first evident from a cursory examination of the opsonic index alone. On the other hand, after reinoculation with the streptococci in these studies, there was a compensatory qualitative change in the phagocytic activity of the granulocytes without an absolute quantitative elevation in their number. In this group a qualitative cellular-humoral response apparently served as a compensatory protective mechanism by which fewer cells, each with greater individual phagocytic capacity, were able to control the infection effectively. Combining both the quantitative (leucocytic) and qualitative (phagocytic) responses, a greater total bacterial phagocytosis was noted following reinoculation with the streptococcus, but the difference with this cellular-humoral response was not as great as would be expected from evaluation of the opsonic index alone.

Wood[12] has recently called attention to the importance of phagocytosis of bacteria by neutrophilic granulocytes in bacterial pneumonia in the absence of demonstrable specific opsonins or antibodies. The motility of the cells in and through the fixed tissues brings direct contact with the invading organisms resulting in ready cytoplasmic inclusion. Our observations strongly suggest the acquisition of a highly specific humoral catalyst, which, upon reinoculation of the same pathogenic agent some months after recovery from the first invasion, greatly facilitates this phenomenon of spontaneous cellular phagocytosis, so that far fewer polymorphonuclears are needed to combat reinfection effectively the second time than the first.

The younger mature cells, according to the Arneth-Shilling index, were definitely more effective in their phagocytic activities in this study, with a decrease in phagocytic capacity as the individual cell age increased. These observations suggest that quantitative phagocytosis bears a direct relationship to the age and motility of the neutrophiles. In the presence of opsonins, the more actively motile "left-shifted" cells have shown the greatest avidity in engulfing reinvading pathogenic bacteria.

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OUTBREAK OF DIARRHEAL DISEASE ASSOCIATED WITH PARACOLON

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ORGANISMS of the incompletely circumscribed paracolon group are felt to be in a controversial position regarding their pathogenicity. A few outbreaks of diarrheal disease in human beings and in animals associated with paracolon flora in the intestinal tract have been recorded,[1-8] although usually it has been impossible to fulfill the postulates of Koch. Similarly, this report concerns an outbreak of diarrhea during which an organism of the paracolon group was isolated from the food consumed and from the stools of thirteen of the sixteen patients who required hospitalization.

In an Army officers' mess, fricasseed chicken was served to about 250 diners from 5:30 to 7:30 P.M. The chicken had been cooked in several large vessels and served directly from them. Approximately 100 servings were made from the one container of chicken which was found to contain paracolon bacteria when cultured later. There were eighty known cases of diarrhea; it could not be determined whether all these cases originated from the same batch of chicken. However, all patients had eaten chicken, and one had eaten nothing whatever except chicken.

About two hours after eating, the first patient became nauseated, had abdominal cramps, and soon began to vomit and pass watery stools. Other cases appeared between three and twelve hours after eating; the largest number of cases occurred four hours after eating the chicken. The five most severely affected patients developed mild shock which was relieved by simple measures. A total of eighty patients reported for treatment; how many were treated elsewhere could not be determined. Sixteen patients required hospitalization; these were the only ones available for culture during the acute illness. After twenty-four hours, all sixteen patients were symptom free except for weakness and had been discharged from the hospital. No sulfonamide therapy was given.

A rectal swab made from each hospital patient about nine o'clock in the morning after onset of the illness was planted on Salmonella-Shigella agar, on eosin-methylene blue agar, MacConkey's medium, and desoxycholate citrate agar and then placed in nutrient broth. Four hours later the nutrient broth was subcultured to eosin-methylene blue agar. No staphylococci were recovered from any of the cultures. In about eight hours, some Salmonella-Shigella agar plates showed heavy growth of lactose-nonfermenters. Almost all plates showed heavy growth of lactose-nonfermenters after twenty-four hours. Colonies were picked at twenty-four and forty-eight hours and, after replating, biochemical characteristics of the isolates were determined. Sugar fermentations in brom-cresol purple-nutrient broth were read daily for fourteen days. Indole forma-

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tion was determined in tryptone water with Kovac's reagent after twenty-four hours of incubation. Citrate utilization was determined on Simmons' medium after twenty-four hours. Motility tests were done in 0.3 per cent agar and were read after five days of incubation at room temperature. Methyl red and Voges-Proskauer tests were done with the M.R.-V.P.* medium. Gelatin liquefaction was read by refrigerating five-day cultures. The urease test of Rustigian and Stuart[9] was used.

The organism recovered from thirteen of the sixteen patients resembled paracolon 29911 of Stuart and co-workers[10] (Table I). It also bears a superficial resemblance to the genus Proteus, although it does not attack urea. It produces a bubble of gas in dextrose broth (which is not revealed after five days' incubation of a double sugar agar slant). Sucrose and mannitol are fermented in two to six days. Lactose, salicin, xylose, maltose, and rhamnose are not attacked in fourteen days. Methyl red and Voges-Proskauer reactions are negative, indole is produced, sodium citrate is utilized, urease is not formed, and the organisms are motile.

TABLE I

	DEXTROSE	LACTOSE	SUCROSE	SALICIN	MALTOSE	MANNITOL	INDOLE	V.P.	CITRATE	UREA	MOTILITY
29911-like strain	AB	–	A_6	–	–	A_2	+	–	+	–	++
29911 (Stuart)	AB	–	A_{5-20}	–	–	–	+	–	+	–+w	++
P. rettgeri	AB	–	AG_s	–	–	AG	+	–	+	+	++
P. morganii	AG	–	–	–	–	–	+	–	–	+s	++
P. vulgaris	AG	–	AG	–AG	AG	–	+	–	±	+	++
P. mirabilis	AG	–	AG	–	–	–	–	±	+	+	++

AB, acid and a bubble of gas; A_6, production of acid in six days of incubation; AG, acid and gas; –AG, some strains produce acid and gas, others do not; +, positive test or reaction; –+w, some strains negative, some weakly positive; +s, slow reaction; –, no reaction or negative test.

This organism differs from the one described by Stuart[10] as 29911 in that sucrose is fermented more rapidly (seven as against twenty days) and mannitol rather than maltose is attacked.

Two of the five strains isolated from the plates prepared from leftover chicken showed properties similar to this typical organism except that one produced a bubble of gas in sucrose broth in thirty days and the other was slow in developing motility.

Nineteen of the cultures from this outbreak (more than one isolate from some patients) have been found to be antigenically identical or very closely related. Further study of these cultures by Stuart has confirmed both biochemical and serologic connections with Type 29911 paracolon (Stuart). These strains may be classified as an atypical 29911 paracolon for the present.[11]

Rapid survey of the other strains isolated shows many related cultures and a large number not so apparently related. In addition to the paracolon described, *Proteus rettgeri* or *Proteus mirabilis* were isolated from twelve of the

*Difco Laboratories, Inc., Detroit, Mich.

sixteen cases. *Proteus mirabilis*, together with the paracolon, was found in the chicken. Moreover, a large number of urease-negative organisms were recovered which differed in one or more respects from the 29911-like paracolon which was most prevalent.

COMMENT

Careful application of standard methods to the study of an outbreak of common diarrheal disease revealed a paracolon as the predominating organism in the food implicated and in the stool cultures of the hospitalized patients. It appears unlikely that a recognized pathogen may have been overlooked. Whether the paracolon or the various Proteus species or the combination of them caused the outbreak cannot be definitely decided. A common pathogen occurring in the same circumstances would have been considered the etiologic agent. *Proteus rettgeri* and the 29911-like paracolon occurred together in several patients; it is noteworthy that the only important difference between the two is the ability of the former and the inability of the latter to attack urea. One is led to speculate as to their relationship, when they are associated in such a violent diarrhea.

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THE EFFECT OF THE RATE OF ADMINISTRATION OF AMINO ACID PREPARATIONS AND BLOOD AMINO ACID NITROGEN LEVEL ON THE PRODUCTION OF NAUSEA AND VOMITING

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THE intravenous administration of protein hydrolysates in the management of various protein deficiency states is now widely accepted. During the infusion untoward reactions occasionally occur which make it necessary to interupt this form of supplementary protein therapy. These reactions include either one or any combination of the following signs or symptoms: Anorexia, nausea, vomiting, flushing, formication, headache, and dizziness.

It has been reported previously that under certain conditions the intravenous administration of casein digests produced a depressing influence on voluntary food intake.[1] In that study no direct relationship was found between the rate of administration and the degree of anorexia. Elimination of the other undesirable side effects could usually be accomplished by reducing the rate of the infusion. Clinically it is not desirable to subject a patient to prolonged infusion requiring three to four hours if it is possible to increase the rate of administrations without ill effects. For example, in order to give large amounts of these preparations by the intravenous route the patient will be required to remain inactive with one arm extended for long periods of time. The possibility of infection occurring at the site of the injection is greatly enhanced by the slow administration of these preparations.

In this hospital three different patients are known to have developed abscesses at the site of infusion, and five others showed clinical evidence of cellulitis following the slow rate of administration of a casein digest.

Hoffman, Kozoll, and Osgood[2] observed that nausea and vomiting occurred in patients who received a casein hydrolysate administered at rapid rates. These investigators are of the opinion that if the plasma amino acid nitrogen level exceeded 10 mg. per cent, nausea and vomiting usually occurred. During the course of previous studies[1] it was shown that a mixture of pure amino acids could be administered at exceedingly rapid rates without causing any undesirable effects.

The present investigations were undertaken to determine whether the relationship of the amino acid nitrogen content of the blood to nausea and vomiting, as proposed by Hoffman, Kozoll, and Osgood, existed with other amino acid preparations. In addition, the effect of the rate of administration on the production of these ill effects was studied.

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METHODS

The patients used in this study were convalescent hospital subjects who had no known metabolic disorders. The intravenous injections were given as though they were a part of their regular therapy; thus the psychologic factors were minimized. The amino acid preparations were administered intravenously at rates varying from 5 to 100 ml. per minute, and if nausea or vomiting occurred a blood sample was immediately withdrawn from the opposite arm for amino acid nitrogen determination. In those patients in whom no symptoms occurred, a blood sample for amino acid content was taken just before the end of the infusion.

Five different amino acid preparations were used in this study. Preparation I* was an enzymatic hydrolysate of casein which was administered as a 10 per cent solution. Preparations II† and IV† were acid digests of casein; the former was a 15 per cent solution and the latter a 6 per cent solution. Tryptophane was added to both Preparations II and IV, and, in addition, methionine was added to Preparation IV. Preparation III‡ consisted of a mixture of the ten "essential" amino acids plus the amino acid glycine. Approximately 50 per cent of the amino acids in this mixture were in the racemic form. Preparation V‡ was a vuj-n-type[3] mixture of amino acids prepared by the recombination and fortification of fractions of an acid hydrolysate of casein. It was administered as a 10 per cent solution. Of the total nitrogen in this mixture approximately 50 per cent was derived from essential amino acids.

Since the relationship between the amino acid nitrogen level of the blood and the presence of nausea and vomiting was one of the major interests of this study, no attempt was made to adjust the nitrogen concentrations of the preparations used. The volume of solution infused was the contents of either a 500 or a 1,000 ml. bottle. In the instance of Preparation II, however, due to the hypertonicity of the 15 per cent solution, 266 ml. of the protein hydrolysate were diluted to 500 ml. with sterile water and the resulting mixture administered.

The determination of the amino acid nitrogen content of the blood was made by the copper method of Albanese, and Irby,[4] all values being expressed as milligrams of amino acid nitrogen per 100 ml. of plasma.

RESULTS

The influence of the rate of intravenous infusion on the occurrence of nausea and vomiting for all these preparations is summarized in Fig. 1. This factor was studied in 115 subjects. The casein digests (Preparations I, II, and IV) all had a high content of glutamic and aspartic acids, whereas the amino acid mixtures (preparations V and III) contained, respectively, little or none of these dicarboxylic acids. There appeared to be a slight increase in the incidence of nausea or vomiting when the rate of administration of preparation

*Preparation I (Amigen, 10 per cent solution) was supplied by Mead Johnson and Company, Evansville, Ind.

†Preparations II (Parenamine) and IV (6 per cent Parenamine) were furnished by the Frederick Stearns & Company, Detroit, Mich.

‡Preparations III (Vuj) and V (Vuj-n-IX) were provided by Merck & Company, Inc., Rahway, N. J.

I was increased from 5 to 15 ml. per minute. In the case of Preparation II or IV, the increased rate of administration was usually accompanied by a marked increase in the incidence of nausea or vomiting. On the other hand, the administration of the amino acid mixtures, Preparation III or V, was accompanied by an exceedingly low incidence of ill effects. In this connection it is noteworthy that Preparation III has never made any patient ill, either in this or in earlier studies carried out in this hospital. The incidence of undesired side reactions was slightly higher when Preparation V was administered. It should be emphasized that the amino acid mixtures, Preparations III and V, were administered at increasing rates which began at the highest tolerated level for the protein hydrolysates. In many cases Preparation III was given at the rate of 500 ml. in eleven to thirteen minutes, whereas Preparation V was administered at the unusually rapid rate of 100 ml. per minute; thus, a 500 ml. bottle of this amino acid mixture was given in five minutes. In some of these cases flushing and formication occurred, but there was no nausea or vomiting.

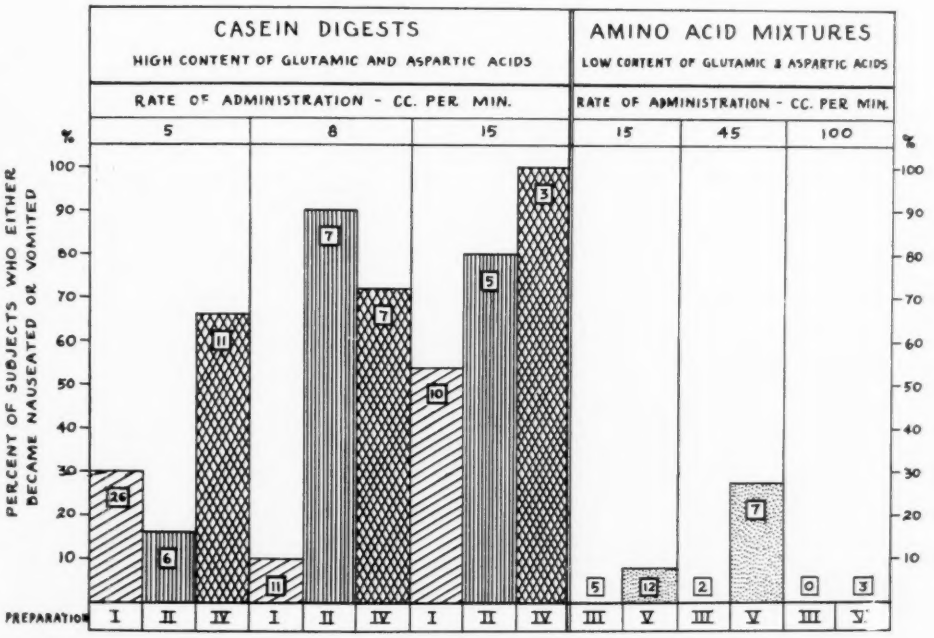

Fig. 1.—Influence of rate of administration on incidence of nausea or vomiting (115 subjects). The numbers in the squares at the top of each column indicate the number of subjects who received one of the five preparations shown along the bottom of the chart.

The relationship between the occurrence of vomiting and the level of the amino acid nitrogen in the blood plasma is summarized in Fig. 2. In this part of the study an attempt was made to vary the amino acid levels of the blood by varying the rate of the intravenous infusion. The administration of Preparation I caused no significant change in the incidence of nausea and vomiting until the amino acid nitrogen content of the plasma reached 13 mg. per 100 ml. or greater, at which levels 50 per cent of the patients became ill. With levels

below this, the frequency of nausea and vomiting ranged from 25 to 30 per cent of the patients studied. The highest amino acid nitrogen level in the plasma attained with this Preparation was 34 mg. per cent and in this subject only nausea occurred. When Preparation II was used, the incidence of nausea and vomiting increased progressively with an increase in the plasma amino acid nitrogen; if the amino acid nitrogen level of the plasma reached 9 mg. per cent or greater, all the subjects either became nauseated or vomited. With Preparation III there was no nausea or vomiting in any of the seven subjects reported in this study. In another series of fifteen patients who received forty-six infusions using this preparation, there also was no occurrence of any ill

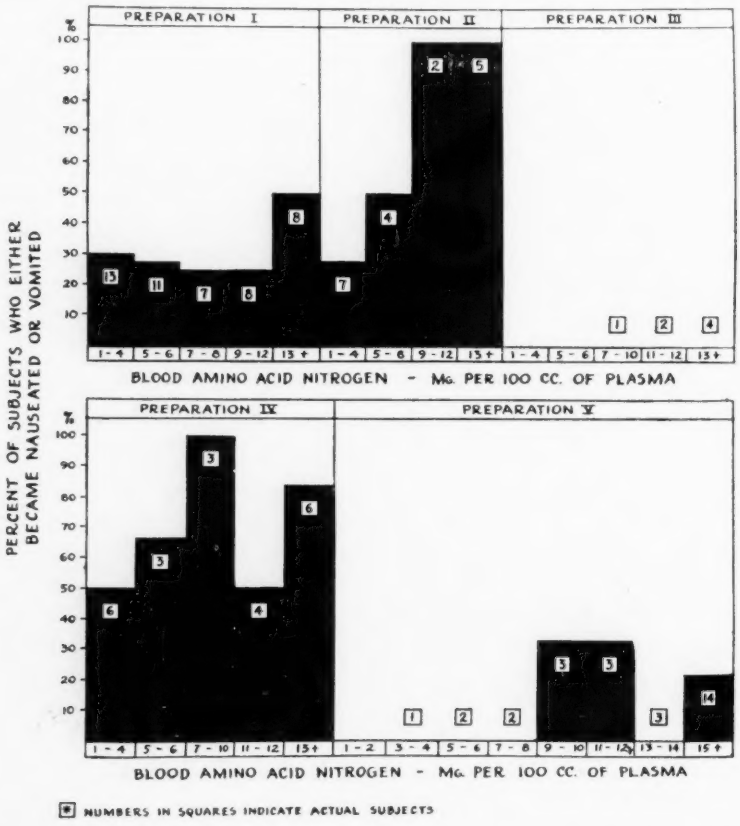
PREPARATION I
PREPARATION II
PREPARATION III
PREPARATION IV
PREPARATION V
PERCENT OF SUBJECTS WHO EITHER BECAME NAUSEATED OR VOMITED
BLOOD AMINO ACID NITROGEN - Mg. PER 100 CC. OF PLASMA
NUMBERS IN SQUARES INDICATE ACTUAL SUBJECTS

Fig. 2.—Occurrence of nausea or vomiting in relation to preparations and blood amino acid nitrogen levels (122 subjects). The small numbers (from 1 to 15 or greater) along the bottom of the charts indicate the range of blood amino acid nitrogen level in mg. per cent attained in the group of subjects directly above.

effect. In a qualitative manner, Preparation IV reacted similarly to Preparation II, though the incidence of nausea and vomiting was not as great. Preparation V was administered at exceedingly rapid rates, and even at the highest plasma amino acid nitrogen level obtained, the incidence of nausea and vomiting was only 20 per cent. An amino acid nitrogen value of 44 mg. per cent was reached in one patient receiving Preparation V with no ill effects whatsoever.

DISCUSSION

From the data presented in this investigation the incidence of nausea and vomiting appears to depend on the amino acid composition of the preparation used rather than on the average rate of administration or on the plasma amino acid nitrogen level. In all patients who received casein hydrolysates (Preparations I, II, and IV) the incidence of nausea or vomiting was greater than in those subjects who received the amino acid mixtures (Preparations III and V). In the case of Preparation II it was found that the presence of an amino acid nitrogen content in the plasma of about 10 mg. per cent or greater was usually associated with the occurrence of nausea or vomiting. This observation is in agreement with the work of Hoffman, Kozoll, and Osgood,[2] who used the same preparation.

The possibility that glutamic and aspartic acids are responsible for these toxic symptoms has been studied in both dogs and human subjects.[5-8] Preparation III was completely devoid of glutamic and aspartic acids and its use parenterally did not produce either nausea or vomiting. Preparations I, II, and IV are produced by hydrolyzing casein, a substance having a high content of these dicarboxylic acids, and therefore these digests are considered to have a large content of glutamic and aspartic acids. The glutamic acid content of Preparation V is 1.25 Gm. per liter or less.* Thus the dicarboxylic acid content of this preparation is considerably lower than in mixtures resulting from the direct hydrolysis of casein. One explanation for the occurrence of nausea and vomiting resulting from the intravenously administered casein digests is that the blood dicarboxylic acid concentration becomes high enough to exceed a nausea threshold. The amino acid Preparations III and V, on the other hand, allow high blood amino acid nitrogen levels to be attained without nausea simply because little or no dicarboxylic acid is being added to the blood stream. Casein digests also differ from the amino acid mixtures by their content of nonamino acid substances which may be an additional factor contributing to the production of these ill effects. The possibility of a correlation between the plasma glutamic acid content and the presence of nausea and vomiting is being investigated.

Any evaluation of the toxic manifestations produced by the parenteral administration of amino acid preparations is difficult because the responses vary widely among apparently normal individuals.

SUMMARY

The effect of five different amino acid preparations on the production of nausea and vomiting was investigated in human subjects. In all patients who received casein hydrolysates intravenously, the incidence of nausea or vomiting was greater than in those subjects who received the amino acid mixtures. Mixtures of amino acids can be administered intravenously at high rates without producing these effects. The casein hydrolysates, on the other hand, must be administered at considerably slower rates to avoid nausea and vomiting.

*Personal communication from Dr. E. E. Howe, Merck & Company, Inc., Rahway, N. J.

With the mixtures of amino acids there appears to be no plasma amino acid nitrogen level at which nausea and vomiting uniformly occur. With casein digests, increased incidence of these ill effects is usually associated with high amino acid nitrogen values. The role of the dicarboxylic acid in the production of nausea and vomiting is discussed.

The production of nausea and vomiting by the intravenous administration of amino acid preparations depends on the preparation used and individual tolerance rather than on the degree of hyperaminoacidemia.

The authors wish to acknowledge the technical assistance of Dorothy L. DeZelia in this study.

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STUDY OF A NEW SEDATIVE-HYPNOTIC DRUG (3,3-DIETHYL-2,4-DIOXOTETRAHYDROPYRIDINE)

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WHILE there is an abundance of sedatives and hypnotics available to physicians, none is completely satisfactory. In fact, even those which are most popular with the profession occasionally elicit untoward side reactions such as drowsiness, "hangover," excitation, and delirium, as well as allergic responses involving chiefly the skin. Such toxic effects are especially encountered with the barbiturates. Thus, there is a place for a mild hypnotic preparation which is distinguished by a minimum of side-reactions and which chemically does not belong to the barbiturates.

In the light of the foregoing it was decided to undertake the clinical appraisal of a sedative-hypnotic compound (3,3-diethyl-2,4-dioxotetrahydropyridine) which appeared to afford certain advantages over the barbiturates, as substantiated by findings of Koppanyi, Herwick, Linegar, and Foster.* These investigators explored the pharmacology of the drug extensively, and some of their observations are summarized here.

The minimal lethal dose of the aforementioned pyridine compound for rabbits was 500 to 650 mg. per kilogram orally and 300 to 350 mg. per kilogram intravenously. In aqueous solution the drug was readily absorbed following oral, intramuscular, subcutaneous, and intraperitoneal administration. The smallest oral dose required in the rabbit for motor paralysis and sensory depression was 250 mg. per kilogram. Sleep was induced within ten minutes when 150 mg. per kilogram were given intramuscularly or intraperitoneally to rabbits, but by the subcutaneous route this dose produced only drowsiness. Motor incoordination, the first sign of effect of the drug, occurred usually within one or two minutes following intramuscular or intraperitoneal injection. In two rabbits receiving daily 250 mg. per kilogram parenterally for seven days, there was no evidence of accumulation. The animals eliminated at least five-sixths of the dose within twenty-four hours. With regard to the duration of action, it was shown that rabbits eliminated, during an average period of five and one-third hours, about 70 per cent of the intravenous fatal dose; that is, about 13 per cent per hour. The average sleeping time from approximately 60 per cent of the minimal lethal dose was found to be as follows: eight hours (rabbits, orally), three and one-half hours (rabbits, intravenously), and five hours (dogs, intravenously). There was great individual variation in recovery time but no evidence of acquired tolerance.

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*Koppanyi, T., Herwick, R. P., Linegar, C. R., and Foster, R. H. K.: Studies on the Pharmacology of 3,3-Diethyl-2,4-Dioxotetrahydropyridine, and 3,3-Diethyl-2,4-Dioxopiperidine, Arch. internat. de pharmacodyn. et de thérap. **64**: 123-152, 1940.

TABLE I

RABBITS ON INTRAVENOUS ADMINISTRATION	3,3-DIETHYL-2,4-DIOXOTETRAHYDRO-PYRIDINE	BARBITAL
Narcotic dose for 50% of animals (ND50) in mg./kg.	86	140
Lethal dose for 50% of animals (LD50) in mg./kg.	350	415

TABLE II

MICE ON SUBCUTANEOUS ADMINISTRATION	3,3-DIETHYL-2,4-DIOXOTETRAHYDRO-PYRIDINE	PHENOBARBITAL
Narcotic dose for 50% of animals (ND50) in mg./kg.	200	222
Lethal dose for 50% of animals (LD50) in mg./kg.	1,062	473

Tables I and II derived from data of the report by Koppanyi and co-workers give some of the comparative results on the experimental pyridine compound, barbital, and phenobarbital.

From these figures it appears that intravenously in rabbits, the narcotic index (LD50/ND50*) of 3,3-diethyl-2,4-dioxotetrahydropyridine is 4.07 and that of barbital is 2.96. Similarly, on subcutaneously administration in mice the narcotic index of the experimental drug is 5.31, but that of phenobarbital is 2.13. It can be concluded that the margin of safety of the pyridine compound ranks decidedly higher than that of either barbital or phenobarbital.

Systemic reactions to 3,3-diethyl-2,4-dioxotetrahydropyridine were similar to those obtained with barbiturates; namely, a fall in blood pressure, depression of respiration and body temperature, and an increase in pulse rate. In a number of other animal experiments it was shown that the pyridine compound behaved typically of a true hypnotic by preventing convulsions from strychnine and picrotoxin. In dogs there was no appreciable effect on the blood sugar except after large amounts of the drug. Rabbits receiving twenty-five to eighty-six daily oral doses of from 15 to 400 mg. per kilogram of the pyridine derivative showed no deviations from normal in general appearance, food consumption, and reaction to other drugs. Likewise, there was no evidence of the development of tolerance, sensitization, or addiction in these animals, and changes in the blood picture were within normal limits.

With this pharmacologic evidence of effectiveness and low toxicity, clinical trials with 3,3-diethyl-2,4-dioxotetrahydropyridine† were instituted.

Obviously, the evaluation of therapeutic results with a sedative-hypnotic is open to a wide range of interpretation since the subjective response of patients to such medication depends upon many factors, some of which are beyond the

*LD50 is defined as the lethal dose for 50 per cent of the animals and ND50 as the narcotic dose which prevents a righting response in 50 per cent of the animals after the tails are pinched.

†3,3-diethyl-2,4-dioxotetrahydropyridine was furnished by Hoffmann-La Roche, Inc., Nutley, N. J., under the designation NU-903, through the courtesy of Dr. Leo A. Pirk.

control of the experimenter. Nevertheless, an attempt was made to evaluate the effects as objectively as possible. Thus relief from complaints was not suggested or promised to the patients, and the reports of responses to the drug were obtained as casually as possible. In fact, the patients were encouraged to volunteer their impressions. Furthermore, a relatively large number of subjects were tested so that many of the potential psychic factors which might influence the drug's appraisal could be neutralized.

NU-903 was administered to two groups of patients: one consisted of subjects suffering from insomnia, and the other of persons requiring daytime sedation because of nervous tension. Among the latter were patients with a variety of psychosomatic disorders.

In the first group there were ninety-six patients who had all complained that they slept poorly or fitfully. Of these, sixty-six received one tablet of NU-903 (0.2 Gm.) at bedtime; forty-two, including twelve who had failed to respond to the aforementioned dosage, were given two tablets (0.4 Gm.), all for at least seven consecutive nights. An uninterrupted period of trial makes changes more perceptible, and thus these patients were in a better position to appraise the effect of the drug on their sleep than if they had taken it only sporadically. The higher dosage was administered in those instances in which clinical appraisal indicated a more severe degree of insomnia or a more agitated mind.

The effects of this treatment are shown in Table III. The degree of the positive responses is indicated by the number of plus marks: three signs denote excellent; two signs, satisfactory; and one sign, fair results. Negative responses are designated by 0.

TABLE III. TREATMENT OF INSOMNIA WITH NU-903

DOSAGE	NUMBER OF PATIENTS	TOTAL NUMBER OF TREATMENT	AVERAGE ONSET OF EFFECT	AVERAGE DURATION OF SLEEP	RESULTS
0.2 Gm.	66	482	30 min.	6 hr.	10 +++
					21 ++
					24 +
					11 0
0.4 Gm.	42	478	30 min.	7 hr.	10 +++
					22 ++
					6 +
					4 0

It is apparent that 0.2 Gm. of NU-903 was not quite sufficient to induce satisfactory sleep in the majority of patients, although it did have a beneficial effect on many. It seems as though the desired therapeutic results were obtained with this dose only in persons who were sensitive to hypnotic drugs or whose insomnia was of a mild degree. Furthermore, it is evident that 0.4 Gm. of NU-903 produced excellent or satisfactory sleep in thirty-two of forty-two patients. (Among those reporting gratifying results on 0.4 Gm. were twelve patients in whom 0.2 Gm. had failed to induce satisfactory sleep.) Six patients claimed to have had only a fair degree of response with 0.4 grams. Finally, of the four subjects

who reported no results with this dose, three had previously experienced insufficient hypnotic effect from 0.1 Gm. of sodium pentobarbital, but they did have a good night's sleep from 0.2 Gm. of the latter somnifacient. From these results it may be concluded that 0.4 Gm. of NU-903 will produce a hypnotic effect comparable to that of 0.1 Gm. of sodium pentobarbital, the standard dose.

The onset of sleep, regardless of the dose, followed taking of the drug usually within thirty minutes, but never after more than sixty minutes. The duration of sleep averaged six hours following 0.2 Gm. of NU-903 and seven hours following 0.4 Gm., with those patients being taken into consideration who reported excellent or satisfactory results.

One subject receiving 0.2 Gm. and one receiving 0.4 Gm. complained of drowsiness upon awakening. Otherwise, there was a complete lack of untoward subjective reactions. Similarly, neither allergic phenomena nor other signs of idiosynerasies were observed. This relative freedom from undesirable side effects offers a distinct advantage over the barbiturates, as evidenced by the fact that among the ninety-six patients treated for insomnia with NU-903 there were six persons who had experienced untoward reactions to barbiturates, such as excitability, excessive drowsiness, or skin reactions.

In the second group receiving NU-903 for daytime sedation, there were eighty-seven patients. About 60 per cent of these took amphetamine sulfate (30 mg. a day) for the purpose of weight reduction, and in these the experimental drug was given to overcome the nervous excitation induced by this stimulant.

Thirty-five patients received one-half tablet of NU-903 three times daily (total dose 0.3 Gm.), and fifty-two were given one tablet three times daily (total dose 0.6 Gm.). At the lower dosage level the average number of treatment days was fifteen, and at the higher dosage level it was twenty-one. However, eighteen patients received 0.2 Gm. three times daily for thirty days, and three for as long as ninety days.

The effects of this treatment are shown in Table IV. The degree of favorable responses was more difficult to ascertain than in the group receiving the drug as a soporific, and the therapeutic effects were therefore listed in a broader manner, by classifying them as satisfactory, fair, or none.

TABLE IV. TREATMENT OF NERVOUS TENSION WITH NU-903

DOSAGE	NUMBER OF PATIENTS	TOTAL NUMBER OF TREATMENT DAYS	RESULTS	
0.1 Gm. t.i.d.	35	525	22	Satisfactory
			10	Fair
			3	None
0.2 Gm. t.i.d.	52	1,092	36	Satisfactory
			10	Fair
			6	None

As can be seen from Table IV, beneficial results were obtained in the majority of patients taking either 0.1 Gm. or 0.2 Gm. three times daily. The incidence of ineffectiveness of the drug as a sedative was about 10 per cent. It is not

easy to compare the responses to NU-903 with those to other sedatives because of the lack of objective criteria for such a comparison. Nevertheless, in eighteen patients who had received 0.2 Gm. of NU-903 three times daily, 30 mg. of phenobarbital, also given three times daily, were substituted for the experimental preparation. Six of these subjects claimed that they felt a greater degree of relaxation of nervous tension when they received NU-903. These observations give the tentative impression that the sedative effect of 200 mg. of NU-903 is somewhat superior to that of 30 mg. of phenobarbital. It may be inferred that on a weight basis, about four to five times as much of NU-903 as of phenobarbital has to be given in order to obtain the same degree of sedation. Untoward subjective reactions were conspicuous by their absence in all eighty-seven patients of this group. Likewise, there were no signs of idiosyncrasy. In this connection it is noteworthy that five of these eighty-seven subjects had previously reacted with a pruritic rash to barbiturate medication.

For ten of the patients receiving 0.1 Gm. of NU-903 three times daily for two weeks (included in Table IV), pre- and postmedication blood counts were done, including red, white, and differential counts, as well as hemoglobin determinations. There was no significant change in the blood picture of any of the subjects. In addition, a collateral study of a similar nature was carried out in nine persons not requiring sedative medication and, therefore, not included in the series that served for clinical evaluation of the experimental preparation. Each of these subjects received 0.1 Gm. of NU-903 three times daily for periods ranging from twenty-two to sixty-one days. The average number of treatment days was 38.2, and the total doses taken by the individual persons during the period of observation were as follows: 18.3, 12.6, 9.9, 12.9, 9.3, 6.6, 12.6, 13.8, and 7.2 grams. Hemograms, including differential counts and hemoglobin determinations, done at initiation and termination of medication revealed that there were no significant changes in the quality of the blood in any of the nine subjects.

SUMMARY AND CONCLUSIONS

A new sedative-hypnotic drug, 3,3-diethyl-2,4-dioxotetrahydropyridine, tentatively designated NU-903, was evaluated clinically in 183 patients. There were two groups of subjects: to one group, consisting of ninety-six patients, close to 1,000 doses of the drug were given to induce hypnosis, and to the other, consisting of eighty-seven patients, the preparation was administered as a daytime sedative for a total of over 1,600 treatment days. The results were as follows:

Four-tenths of a gram produced excellent or satisfactory sleep in thirty-two of forty-two patients. A dose of 0.2 Gm. proved less gratifying as a somnifacient, judged by results recorded for sixty-six patients. Of these, only thirty-two responded with excellent to satisfactory sleep, and the rest had poor or no results. However, in twelve in whom the lower dosage failed to produce gratifying results, satisfactory sleep was induced by 0.4 grams. In all subjects medication was given for at least seven consecutive nights.

For treatment of nervous tension, 0.1 Gm. of NU-903 three times daily was prescribed to thirty-five patients (average number of treatment days, fifteen),

and 0.2 Gm. three times daily to fifty-two individuals (average number of treatment days, twenty-one). Adequate sedation was obtained at both dosage levels in the majority of patients; there was no sedative effect in 10 per cent of the cases.

One subject receiving 0.2 Gm. and one receiving 0.4 Gm. as a somnifacient complained of drowsiness upon awakening. Otherwise, that is, in 181 patients, there was complete absence of untoward subjective reactions. No allergic phenomena were observed in any of the 183 cases treated. In contrast, eleven of these subjects experienced undesired aftereffects following barbiturate medication.

Blood studies carried out in nineteen subjects receiving 0.1 Gm. of NU-903 three times daily over prolonged periods of time did not reveal any untoward effects of the drug on the quality of the blood.

It can be concluded that NU-903 is an effective hypnotic and sedative which is distinguished by excellent tolerability.

LABORATORY METHODS

THE ASSAY OF HEPARIN

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NUMEROUS methods for the standardization of heparin using citrated or oxalated whole blood or plasma have been described in the literature. Because of peculiarities in the development of clots in vitro, and the difficulty in distinguishing one from another, several means of determining an end point have been suggested. In Foster's[1] modification of the Reinert and Winterstein method, as many as eleven evaluations for various clot formations, ranging from a full clot to no clot, have been proposed. The assigned values are plotted logarithmically to determine the theoretic one-half clot. The method of Howell, as modified by Charles and Scott,[2] calls for the use of cat blood. Clot formations occurring in the standard heparin tube series are matched against those seen in the unknown series. Large differences in the clotting nature of cat blood renders difficulty in duplicating results. The method of Jacques and Charles[3] utilizes oxalated beef blood with the addition of thrombin. Impurities in heparin samples interfere with the clotting mechanism and usually have a tendency to exaggerate their anticoagulant activity. The Fischer and Schmitz[4] procedure using chicken blood does not permit the repetition of the assay under identical conditions. Kuizenga, Nelson, and Cartland[5] report that recalcified sheep plasma requires a smaller increment in heparin for a transition from coagulation to fluidity than either recalcified beef or horse plasma. They report that 2 μg (0.2 Toronto unit) of standard heparin containing 100 Toronto units per milligram are necessary for this complete change. In the method herein described, only 1 μg of such standard heparin is required to effect this change for recalcified beef or sheep plasma.

The evaluation of clots according to opacity, granulation, density, adherence to the walls of test tubes, traces, etc., is difficult. Values so assigned are subject to personal error of judgment and can lead to an erroneously plotted or matched end point. Experience has shown that two persons seldom evaluate or match clots identically. Also, slight modifications in the amounts of calcium, plasma or whole blood, saline, temperature, or time affect the development of clots to a noticeable degree. My experience is that a difference of as little as 0.05 mg. of calcium or 0.1 μg of heparin between two tubes containing 1.0 ml. of diluted citrated plasma or whole blood can cause an appreciable difference in clot formation. Such slight errors can easily be made in an assay, and their combined effects may be additive in determining the development of a clot.

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My several years' experience with Foster's method, while working with him, has indicated possibilities for a simplified and improved heparin assay which are incorporated in the method herein described. The increment of heparin in each tube in a series is very small and constant, since only 1.0 μg. of the standard heparin is necessary to pass from clot to fluidity. The time which elapses between the addition of the calcium to the plasma is reduced to a minimum so that all tubes are treated identically. The end point is the least amount of heparin necessary to prevent any clot formation; it is definite and reproducible. Fresh or frozen[6] citrated plasma can be used with equal accuracy.

PROCEDURE

Plasma.—Fresh beef blood is obtained in any desired quantity at the slaughterhouse. Flasks for holding the blood are prepared in the laboratory by placing therein 100 ml. of an 8 per cent sodium citrate solution and marking them at a 2-liter level. The blood is collected from the animals in paraffin-coated pitchers and immediately poured into the flasks to the 2-liter mark. The flasks are then stoppered and agitated gently. Upon reaching the laboratory, the blood is centrifuged at 2,000 r.p.m. for thirty minutes and the supernatant plasma is siphoned off and pooled. Each heparin sample to be assayed requires 12 ml. of plasma. Therefore, quantities slightly in excess of the amount required for an assay consisting of the standard heparin and any desired number of heparin samples are filled into Florence flasks. The flasks should be filled to approximately two-fifths of their capacity. They are then corked, frozen rapidly by rotation in a tilted position in an acetone dry ice bath, and stored in a refrigerator at –20° C.

Calcium Chloride Determination for Clotting.—The optimum calcium chloride level for recalcifying the plasma in this procedure must be determined for each new lot of fresh or frozen citrated plasma. This level is the amount of calcium chloride necessary to permit the end-point tube (the tube in which any clot formation has just been prevented by the heparin) in the standard heparin series to be located near the middle of the series. This quantity of $CaCl_2$ must be contained in a 0.2 ml. volume of physiologic saline which is used for each tube throughout the assay. The standard heparin should contain at least 100 Toronto units or 500 anticoagulant units[7] per milligram.

When blood is citrated as previously described, usually 0.95 mg. of calcium chloride is necessary for the recalcification. However, the amount may vary slightly and can be determined in the following manner: To six tubes containing 0.35 ml. of a 10 μg per milliliter standard heparin solution is added, from the first to the last, 0.16, 0.18, 0.19, 0.20, 0.21, and 0.22 ml., respectively, of a 5 mg. per milliliter calcium chloride solution. Saline is added to bring the volume to 1.0 milliliter. Add 1.0 ml. of plasma and stopper with corks which have only the inserted face coated with paraffin (this prevents the corks from slipping out), invert two or three times, and place in a water bath at 37° C. for three hours. Then examine the tubes and select the one with the

most amount of calcium chloride having no clot formation. The amount of calcium chloride added to that tube is to be contained in the 0.2 ml. volume of physiologic saline mentioned. The concentration so determined can be used for all subsequent assays, using portions of the same plasma.

Assay.—The standard heparin powder and unknown finished powders are made up in concentrations of 10 μg per milliliter. Impure heparin powders, generally low in potency, are made up in concentrations of 20 or 40 μg per milliliter, depending upon their activities. One or two assays may be necessary to determine the concentration to use. Heparin vial solutions are diluted to a concentration of 1.0 Toronto unit per milliliter. Physiologic saline is used for all solutions. Acid-cleaned glassware is a prerequisite for an accurate assay. A series of twelve tubes (10 by 100 mm.) is used for the standard heparin and for each sample to be assayed. Microburettes are used for measuring the plasma and all solutions. To the first tube of the standard heparin series is added 0.23 ml. of the standard heparin solution; in each subsequent tube the amount of heparin solution added is increased by 0.02 ml. over the preceding tube so that the twelfth tube will contain 0.45 milliliter. Each unknown heparin solution is likewise measured into a series of twelve tubes. Saline is then added to all tubes to bring the volumes to 0.8 milliliter. A constant sequence is kept in adding the plasma and calcium chloride solution. To the first tube of each series add 1.0 ml. of plasma and 0.2 ml. of the calcium chloride solution; stopper with paraffin-coated corks, invert two or three times, and place in a water bath at 37° C. for three hours. Repeat this procedure for the second tube of each series and each subsequent tube until all the tubes have been so treated. By doing this, the influence of plasma changes is minimized. After three hours, the tubes are observed. That tube in each series in which any clot formation has just been prevented is the end-point tube for that series. The use of fluorescent light has been found most desirable for the detection of small clot formations. Very often a thin film of clot formation adheres to the rounded bottom of the tube; this is detected by inverting and uprighting the tubes rapidly once or twice in front of the lamp. The amount of heparin in the end-point tubes is used to calculate the potency of the unknown heparin represented by that series, in terms of the standard heparin.

Example.—The standard heparin potency is 110 Toronto units per milligram. To prevent clot formation, 3.1 μg of the unknown heparin powder and 2.7 μg of the standard heparin are required; the strength of the unknown heparin is 95.8 Toronto units per milligram.

$$\frac{110 \times 2.7}{3.1} = 95.8 \text{ Toronto units per milligram.}$$

If in this example, the unknown represented a heparin vial (10 mg. per cubic centimeter), diluted 1,000 times, the potency of 1.0 ml. of the original vial solution would be

$$\frac{110 \times 2.7}{3.1} \times 10 = 958 \text{ Toronto units per milliliter.}$$

DISCUSSION

The small increment of heparin permits an end point to be approached with a greater degree of accuracy than has heretofore been possible in other heparin assays. The increment makes it possible to detect with ease slight errors in titration in following the procedure because the diminution of the clot to fluidity is remarkably uniform in the series. This transition range, under the specific conditions of the assay procedure presented in this paper, requires only 0.1 Toronto unit of activity (1 μg of standard heparin). Fresh and frozen citrated beef and sheep plasma have been used with the same degree of accuracy, and the transition from clot to fluidity requires approximately the same heparin activity. It is, however, easier to determine traces of clot formation in beef plasma than in sheep plasma. Also, it is easier to obtain beef plasma, in large quantities, than sheep plasma, and the results obtained are more uniformly duplicated. Repeated tests on various concentrations of the standard heparin used in this laboratory yielded results within $\pm$ 10 per cent of the theoretic potencies.

SUMMARY

1. A method for the assay of the anticoagulant activity of heparin using recalcified fresh or frozen beef or sheep plasma has been presented.

2. The end point is definite.

3. The method is rapid, simple to execute, reproducible within less than 10 per cent error, employs simple calculation for potency determination, and enables the repetition of the assay using the same material under similar conditions of assay.

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PERMANENT METACHROMATIC STAINING OF MUCUS IN TISSUE SECTIONS AND SMEARS

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THE cytology of mucous secretion in the digestive tract has been neglected as a field of investigation in gastroenterology, in part because of the lack of a satisfactory staining method for the mucins. The only stain for this purpose which has withstood the test of time is mucicarmine, and this has been reliable only for mucus in typical goblet cells. In the stomach, where the mucus normally occurs in the columnar surface epithelium and the neck chief cells, its reliability is notably poor. Thionine and toluidine blue are examples of another type of mucin stain. These dyes differentiate sulfuric acid esters of high molecular weight (for example, the mucins) from other tissue components by a characteristic bichromatic effect called metachromasia, and thereby obviate the need for counterstaining for this purpose. In the case of toluidine blue the mucins are stained some shade of red or purple, whereas other cellular components are stained blue. In addition to this advantage, the metachromatic stains are superior to mucicarmine in respect to their sensitivity and specificity. For these reasons, toluidine blue or thionine would long ago have become the preferred mucin stain were it not for the fact that their characteristic bichromatic effect is lost as soon as the stained tissue is subjected to any dehydrating procedure.

Some time ago we[3] reported a technique for permanent metachromatic staining which was suitable for smears of viscous gastric mucus but not for tissue sections. When this method was applied to smears of secretion from gastric pouches stimulated with mustard oil, or to smears of human gastric juice, it often resulted in the loss of most of the material during the staining process. Although tissue sections were stained effectively, the metachromatic effect was transient. Furthermore, smears treated in this way frequently showed disintegration of the cytoplasmic portion of the cells, but the nuclei were well preserved.

The present investigation was undertaken in an effort to overcome these major deficiencies. To this end, it was necessary to reconsider every step in the procedure and to test it repeatedly on a variety of tissues from animals of different species, as well as on mucus from dog and man.

Fixation.—Smears of mucous material gave excellent results after simple air drying, without chemical fixation. For tissues, a number of fixatives were tried without success. Ten per cent formalin failed to preserve the mucus effectively and stained connective tissue red as well. After fixing with Helly's

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fluid the tissue took the dye, but poorly. A saturated solution of mercuric chloride led to considerable shrinkage and caused precipitation of the dye. A saturated solution of mercuric chloride containing 5 per cent acetic acid gave irregular, granular staining. Alcohol-formol or 70 per cent alcohol alone prevented staining of mucinous substances altogether. The one fixative which gave truly satisfactory results was Zenker's solution with acetic acid, prepared without the addition of sodium sulfate. Neither potassium bichromate nor mercuric chloride alone yielded the same degree of color intensity and brilliance as a mixture of the two. This fixative preserved both intra- and extracellular mucin and augmented the contrast between the metachromatic and normochromatic structures without inducing a diffuse metachromatic staining of the connective tissue. Another advantage was preservation of cytologic detail. Toluidine blue is in general an excellent nuclear stain, but its efficiency in this regard is markedly increased by Zenker's fixation.

Staining.—After repeated trials under a variety of conditions, the staining solution finally adopted contained 0.25 Gm. of toluidine blue per 100 ml. of 0.25 per cent borax solution. When the dye was more dilute than this, it did not stain deeply enough, whereas more concentrated solutions led to a diffuse purple staining of all structures with practically no bichromatic differentiation. The pH of the staining solution is fixed about 9.0 by the borax. It was found that most of the tissues could be stained well also with a simple aqueous solution of the dye (pH 5.5), except for the surface epithelium of feline and human stomachs. In these two instances, the intracellular mucus failed to take the dye unless the pH of the staining solution was adjusted to 9.0, a value which was found to be adequate for all the other tissues and all the smears. After the usual processing of the paraffin sections from xylol to water, it was found desirable to rinse the slides in a fluid of about the same pH (for example, 0.25 per cent borax) both before and after staining. Smears are rinsed only after being stained. The optimal staining time was thirty seconds for all materials examined. Any prolongation beyond this limit leads to overstaining and a nonspecific purple coloration. There is a tendency for the connective tissue to be stained metachromatically at this stage, but this usually disappears during subsequent treatment.

Preservation of Metachromasia.—Ever since the investigations of Hoyer[5] it has been known that the metachromatic effect disappears as soon as the stained sections are treated with alcohol. Attempts have been made to overcome this difficulty by mounting the water-wet preparation directly in a glycerine-containing medium or in a levulose syrup[9]; also, by dehydrating with acetone instead of alcohol[7] before treatment with xylol for mounting in balsam. All of these techniques failed to give us the desired permanence, as did a multitude of other empiric procedures. It was finally found that metachromasia can be preserved if the stained and washed section is treated with an aqueous solution of 0.5 per cent potassium bichromate, saturated at room temperature with mercuric chloride.

Dehydration and Mounting.—Following this treatment with bichromate-sublimate solution, the slide is washed with absolute alcohol. If other than

anhydrous alcohol is used, connective tissue, smooth muscle fibers, and even some nuclei acquire a red coloration. It also happens that an occasional finished preparation shows streaks of blue dye. Both of these irregularities can be prevented if each slide is rinsed thoroughly with a stream of fresh absolute alcohol from a siphon bottle, instead of being immersed in a staining dish containing alcohol which has become diluted from repeated use. Omission of the usual treatment with graded concentrations of alcohol does not lead to any considerable shrinkage. At this stage of the procedure the section shows excellent metachromasia, but when it is brought into xylol all its elements acquire a bluish coloration. With this reduction in reddish tones the normochromatic elements become a purer blue, but there is a loss in sharpness of the metachromatic differentiation. Such purification of color tone in the normochromatic elements is distinctly advantageous, and it can be retained even when the metachromasia is reintensified by the next step; that is, retreatment with absolute alcohol followed by the bichromate-sublimate mixture. The section is then washed with absolute alcohol containing 1 per cent of rosin, which intensifies the red shades with toluidine blue as it has been shown to do with Giemsa stain. The slide is then carried through two changes of benzol and mounted in Canada balsam dissolved in benzol. The use of xylol, toluol, or acetone for clearing or as solvent for the balsam must be avoided because they destroy the metachromasia. Clarite may be substituted for benzol-balsam, but the results are less satisfactory.

OUTLINE OF THE STAINING TECHNIQUE

Tissues are fixed in Zenker's solution containing 5 per cent acetic acid, and the paraffin sections are carried through to water in the usual way. They are then rinsed in 0.25 per cent aqueous borax solution and drained free of surplus fluid. Smears are prepared from mucus specimens immediately after collection and are left to dry at room temperature for the *minimum* time required. This is usually two to four hours. Mucus smears are not given the preliminary rinsing in borax solution. Slides of both smears and tissue sections are then treated as follows:

1. Cover for thirty seconds with ample staining solution (0.25 Gm. toluidine blue 0* dissolved in 100 ml. of 0.25 per cent borax solution).
2. Rinse with 0.25 per cent borax solution.
3. Cover for fifteen seconds with a 0.5 per cent solution of potassium bichromate, which has been saturated with mercuric chloride at room temperature and filtered, and drain.
4. Cover the slide for two minutes with a fresh portion of the bichromate-sublimate solution and drain.
5. Wash thoroughly for about ten seconds with a stream of fresh absolute alcohol from a siphon bottle.
6. Immerse in xylol for thirty seconds.
7. Rinse again with absolute alcohol and drain completely.

*National Aniline, Certified.

8. Cover again with the bichromate-sublimate solution for thirty seconds.
9. Rinse thoroughly with rosin alcohol (1 Gm. rosin, U. S. P., dissolved in 100 ml. absolute alcohol) and drain.
10. Carry through two changes of pure benzol (one minute each).
11. Mount in Canada balsam dissolved in benzol.

DISCUSSION

In order to assure ourselves of the permanence of the metachromatic differentiation obtained by means of the foregoing procedure, it was applied to a variety of tissues in May, 1945. For this purpose specimens from stomach, jejunum, colon, salivary glands, and bronchi were obtained from the dog, cat, rat, guinea pig, and rabbit. Specimens of resected human stomachs, taken at operation, and guinea pig cervix were also included, as were smears of mucus collected from Heidenhain pouch dogs and human stomachs under eugenol stimulation.[4] At the time of this report, two years after preparation, these slides continue to show excellent preservation of metachromatic as well as normochromatic colors. Only in the goblet cells of rat jejunum is their any evidence of a loss of metachromasia, but even in this instance some areas of the section still contain purple-stained material.

Various theories have been advanced to account for the metachromatic properties of certain dyes like toluidine blue.[1-3, 4a, 6, 8] Recently, Michaelis and Granick[9] added quantitative spectrophotometric evidence to support the view that the shift from blue to purple is due to polymerization of the dye molecules. The simple monomeric form possesses the normal color (pure blue), whereas the polymers are purple to pink, depending on the degree of polymerization. Increasing the concentration of dye induces a shift in color toward the purple, even in the absence of a metachromatically staining substance,[10] and this change is completely reversible on subsequent dilution. This effect is readily explained by the polymerization theory. Alcoholic solutions of the dye show only the blue coloration, and treatment of metachromatically stained substances with alcohol invariably leads to a disappearance of metachromasia. This reversal can be prevented by the action of a mixture of potassium bichromate and mercuric chloride on the metachromatic complex of dye and substrate. As yet, no explanation has been offered for either the deleterious effect of alcohol on metachromasia or the stabilizing effect of the bichromate-sublimate mixture. It is interesting, however, that treatment with the latter does not induce metachromatic staining of previously normochromatic structures; it merely preserves and improves the effects which are already existent.

The superiority of the present technique over that previously reported by us may be ascribed to the change in pH of the staining and washing fluids from an acid value (circa 3) to an alkaline one (circa 9). As a result, cytoplasmic destruction in smears is eliminated and loss of material from the slide is prevented. Although the success of this method in tissue sections depends largely upon this different pH, it is the use of the bichromate-sublimate mixture which effects the permanence of the metachromasia. On the other hand, the present

procedure possesses certain obvious disadvantages. Occasionally the finished preparations of smears, but not of paraffin sections, contain residues of bichromate which give a disturbing yellow-green coloration in some areas, and as yet we have not been able to eliminate this. Also, the method is applicable only to tissues which have been fixed in Zenker's solution; if it could be used after formalin and Bouin's fluid, this would be a decided advantage for the purposes of the routine pathology laboratory. We hope that these difficulties may also be overcome in time and that the entire procedure may be simplified.

We wish to express our thanks to Dr. B. P. Sonnenblick, who stained a great variety of tissue sections and smears by this method in order to confirm our experience with it.

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RETICULUM STAINING WITH SCHIFF REAGENT AFTER OXIDATION BY ACIDIFIED SODIUM PERIODATE

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THE method to be reported herein was evolved from studies on the relation of the Bauer[1] glycogen method to the Jackson-Hudson[2] periodate oxidation of polysaccharides. These studies are being published elsewhere in extenso.

Glycogen, epithelial mucin, cellulose, starch, yeast, and mold chitin and cartilage matrix are stained purplish-red by the Bauer technique.[3] When an acid sodium periodate solution is substituted for the chromic acid of the Bauer method, reticulum of certain parenchymatous organs, notably lung, kidney, thyroid, and cerebral capillaries, is stained a brilliant purplish-red; collagen and fibrin, pink. Capsular substance of certain bacteria and certain elements in Actinomyces granules also stains pink, but further detailed studies are necessary on this.

The technique employed is as follows:

1. Thin (5 μ or less) paraffin sections of routine material fixed in formalin, Orth's fluid, or other fixatives are deparaffinized and brought to 100 per cent alcohol as usual.
2. Soak five minutes in 1 per cent collodion in 50:50 ether and 100 per cent alcohol mixture. Drain one minute with slides held vertically, and harden five minutes, or as much longer as convenient, in 80 per cent alcohol.
3. Wash in water and immerse for ten minutes in sodium periodate (Na_3IO_5), 1 Gm.; 70 per cent nitric acid (specific gravity, 1.42), 0.5 c.c.; distilled water, 100 cubic centimeters.
4. Wash five minutes in running water.
5. Immerse for fifteen minutes in Schiff reagent,† agitating at three- to five-minute intervals, preferably by pouring the fluid out and back.
6. Transfer directly to three successive baths of one and one-half minutes each in 0.5 per cent sodium bisulfite ($NaHSO_3$). (This is slightly weaker than Bensley's[4] M/20.)

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†The Schiff reagent is made as follows: Dissolve 1 Gm. basic fuchsin in 100 c.c. distilled water at 90 to 95° C.; filter while cooling at about 60 to 70° C. When cool, and 2 Gm. sodium bisulfite ($NaHSO_3$) and 20 c.c. normal hydrochloric acid; stopper tightly; put away in cool, dark place overnight. In the morning the solution should be somewhat brownish-yellow in color. Add 300 mg. of finely ground charcoal, shake for one minute, and filter. The solution should become pale yellow. Store in refrigerator at 0 to 5° C. The solution may be used for several weeks, at least if kept cold. Discard if a pink color appears.

7. Wash ten minutes in running water.
8. Stain five to six minutes in Weigert's[5] acid iron chloride hematoxylin. Wash in water.
9. Counterstain one minute in saturated aqueous picric acid solution or in 1 per cent orange G.
10. Dehydrate with alcohols and clear in one change of xylene and 100 per cent alcohol mixture (50:50) and two changes of xylene; mount in clarite.

The Weigert fibrin routine may be introduced in place of steps 9 and 10.

a. Stain in Conn's[6] ammonium oxalate crystal violet (or any other aqueous or hydroalcoholic crystal violet) for two or more minutes.
b. Wash off quickly with 0.9 per cent sodium chloride solution.
c. Treat with the Weigert-Lugol[7] iodine solution (1 Gm. iodine, 2 Gm. potassium iodide, 100 ml. distilled water) for thirty seconds.
d. Blot dry with hard filter paper and decolorize with several successive changes of a few drops each of aniline and xylene (50:50) mixture until violet color ceases to come out.
e. Wash in two or more changes in xylene; mount in clarite.

RESULTS

By all three variants discussed, pulmonary reticulum, renal reticulum, and glomerular stroma, the reticulum of intestinal smooth muscle, the subepithelial basement membrane, the supporting stroma of the cerebral capillaries, and the reticulum of the gastric mucosa were stained a bright purplish-red. Collagen stains pink. Fibrin, hyaline droplets in renal epithelium, hyaline casts, and serous coagula in pulmonary alveoli also stain dull pink of a variable intensity when no plasma stain or the picric acid or orange G variants are used. With the Weigert fibrin variant, fibrin, hyaline droplets, and serous coagula vary from pink to violet, and red corpuscles stain gray to blue-violet. Orange G tinges the erythrocytes orange-yellow but has little other evident effect. Picric acid gives bright yellow erythrocytes and yellow to brown cytoplasm, and gives a yellow overcast to the pink of fibrin, serum, and the like.

With all variants, cartilage matrix is stained red-purple; epithelial mucin, reddish-purple to almost violet; glycogen, dark red-purple to almost black. If confusing, the glycogen may be removed by interposing the Lillie-Greco[8] diastase digestion technique between Steps 1 and 2.

McManus[9] reported the use of a periodic acid Schiff technique for the demonstration of mucin, and recently presented the extension of this technique to the demonstration of renal capillary stroma.*

*At the American Association of Pathologists and Bacteriologists, May 17, 1947, Chicago, Ill.

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THE QUANTITATIVE DETERMINATION OF AMPHETAMINE

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A SATISFACTORY and shortened method for the quantitative recovery of amphetamine from human organs was evolved following repeated experiments in vitro.

Because of the ever-widening use of beta-phenylisopropylamine, variously sold under the name of Amphetamine, Benzedrine, Elastonon, Isoamin, Necodrin, and Orledrin, a study was undertaken to obtain an efficient method of quantitative determination of amphetamine. A technique of extraction is described, using a sulfuric-tungstic acid filtrate, as well as a method of quantitative determination; these greatly reduce the time element in the separation of amphetamine from human organs.

We have used a new technique of approach, employing a combined method of purification, distillation, and diazotization, which is presented in detail.

METHOD

To 25 Gm. of blood, urine, or finely minced organs in a 1-liter flask, add 150 ml. of distilled water. Shake well for five minutes and allow to stand for ten minutes longer. Add 10 ml. of 10 per cent NaOH and shake well for five minutes; then add 30 ml. of 10 per cent sodium tungstate and shake well. Next add slowly 30 ml. of 1 N-H_2SO_4 with continuous shaking, and finally acidify with 18 N-H_2SO_4 and add 5 ml. in excess. Allow to stand for fifteen minutes and filter through a No. 12 Whatman folded filter paper. Transfer the filtrate to a 1-liter round-bottomed flask and make neutral with 10 per cent NaOH; then add 1 ml. in excess. Steam distill through a short-neck flask connected to a Liebig condenser and collect 200 ml. of distillate in a flask containing 10 ml. of 4 NH_2SO_4. Transfer the distillate to a 500 ml. extraction flask, add 20 ml. of ether and make alkaline with 10 per cent NaOH and add 1 ml. in excess. Shake the flask for one minute and extract with three more portions of ether. Combine the ether extracts, wash with water, and discard the water. Extract the ether layer with three 10 ml. portions of 0.5 per cent HCl. Evaporate the HCl extract to dryness over steam bath and diazotize as follows:

Take up residue with 1 ml. of water and add 5 ml. of cold diazotizing reagent (keep volume below 2 ml. for a larger volume of water causes a reduction of color due to failure of complete reaction with diazotizing reagents). Then add 5 ml. of 1.1 per cent sodium carbonate drop by drop with continuous mixing and allow to stand for fifteen minutes. Next add 1 ml. of 10 per cent NaOH drop by drop with continuous mixing and allow to stand for ten minutes. Dilute with water to 40 ml. and mix. Add 10 ml. of n-butanol and mix by inverting. After colored layer has cleared, read transmission in spectrophotelometer at 530 mμ and calculate the amount of amphetamine present from a standard curve.

Nicotine and pyridine do not interfere with the above separation. The amphetamine is identified in an aliquot part of the distillate by obtaining the melting point of the picrate (142° to 143° C.) and the picrolonate (195° to 196° C.).

From the Coroner's Toxicological Laboratories.
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REAGENTS[1]

1. p-Nitroaniline Hydrochloride
   To 0.69 Gm. p-nitroaniline in a 125 ml. glass-stoppered Erlenmeyer flask, add 3 ml. of concentrated HCl. Mix by twirling and break larger lumps with a glass rod. Let stand for ten minutes from the time of addition of the acid. Add 97 ml. of distilled water, a little at a time, with continuous mixing. Stopper the flask and shake vigorously. Filter on a 9 cm. Whatman No. 4 paper, collecting filtrate in a 125 ml. glass-stoppered Pyrex bottle. This solution should be stable at room temperature for at least a year.
2. 0.7 Per Cent Sodium Nitrite ($NaNO_2$)
   Dissolve 0.7 Gm. reagent grade $NaNO_2$ in distilled water and dilute to 100 milliliters. This solution should be refrigerated at all times and should not be used after it is more than one week old.
3. Diazo Reagent (p-Nitrobenzenediazonium Chloride Solution)
   To 5 ml. p-nitroaniline hydrochloride in a 100 ml. glass-stoppered volumetric flask, add 1 ml. of concentrated HCl. Place the flask in an ice-salt bath for ten minutes and then add 3 ml. of 0.7 per cent solution of sodium nitrite. Let stand in ice-salt bath for six minutes, dilute to volume with distilled water (room temperature), mix, and return to ice-salt bath for ten minutes. The reagent is now ready for use and will keep at 0 to 4° C. for at least two weeks under these conditions. It should be tested with amphetamine sulfate standard before using it with unknowns.
4. 1.1 Per Cent Sodium Carbonate (Na_2CO_3) Anhydrous
   Dissolve 2.75 Gm. Na_2CO_3 (reagent grade) in distilled water and dilute to 250 ml. in a Pyrex bottle.
5. 10 Per Cent Sodium Hydroxide
   Made to contain 10 Gm. NaOH in 100 ml. solution; store in a rubber-stoppered 250 ml. Pyrex bottle.
6. n-Butanol
7. Standard Amphetamine Sulfate*
   Made to contain 0.03 mg. amphetamine sulfate per cubic centimeter.

Beyer and Skinner[1] described a new method for amphetamine determination in urine. By diazotizing the extracted amphetamine with p-nitrobenzene, they obtained a red color which was intensified by shaking out with n-butanol.

Gad[2] described a distillation method for tissues. His distillate was collected in 25 ml. of 4 N H_2SO_4. The distillate was made alkaline, then extracted with benzol, and the benzol portion then extracted with 0.02 N HCl. The unused portion of the acid was titrated with 0.02 N sodium hydroxide using methyl red as indicator. Gad also used the spectrophotometer in estimating the amount of amphetamine. The objection to his method was the length of time required and consequent tediousness, as a result of its troublesome foaming.

Using Gad's method, we found that post-mortem material, amphetamine-free, educed a color reaction when tested by the Beyer and Skinner method. We then subjected our distillate, using a sulfuric-tungstic acid filtrate, to the Beyer and Skinner method of estimation of amphetamine, and the result was a colorless blank. With known quantities, a well-defined color was obtained, which could be compared with suitable standards.

Table I is presented to demonstrate that amphetamine is quantitatively recoverable from the liver.

*Smith, Kline and French Laboratories.

TABLE I. RECOVERY OF ADDED AMPHETAMINE SULFATE FROM 25 GM. OF LIVER

EXP.	NAOH CONC.	MG. OF AMPHETAMINE SULFATE ADDED	ML. DISTILLED	MG. RECOVERED	% RECOVERY
1	10%	1.0	250	0.20	20
2	5%	1.0	600	0.43	43
3	1 ml. of 10% NaOH in excess	1.0	100	0.98	98
4	1 ml. of 10% NaOH in excess	2.0	200	1.80	90
5	1 ml. of 10% NaOH in excess	1.0	200	0.95	95
6	1 ml. of 10% NaOH in excess	1.0	200	0.95	95

The precipitated protein residue was tested for the presence of amphetamine with negative results. The distillation was conducted at a pH of 8.

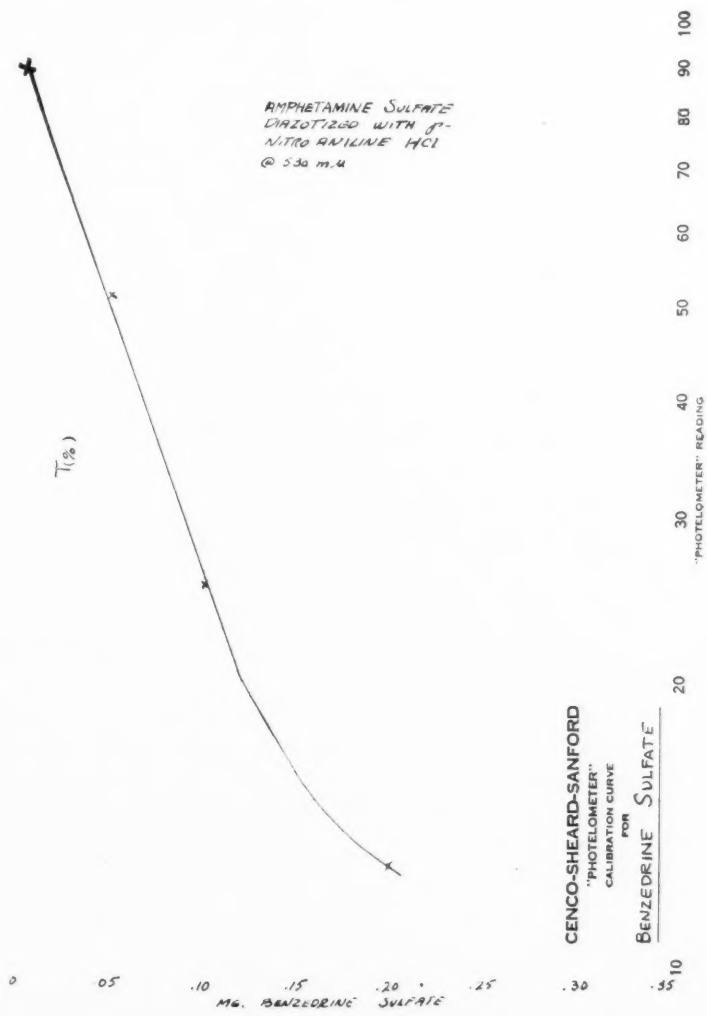

Fig. 1.

Many other investigators have advanced methods of amphetamine distillation. Among them was Schoen,[3] who distilled a tablet into standard sulfuric acid and titrated the unused acid. Keenan[4] described a gold chloride microprecipitation method. Reznek[5] extracted solutions of amphetamine from alkaline solutions with chloroform. The chloroform was extracted with 0.1 N H_2SO_4, and the unused acid was titrated with 0.1 N alkali (1 ml. of 0.1 N acid was equal to 0.0135 Gm. of amphetamine). Dultz[6] used a method for detecting beta-phenylisopropylamine, and beta-phenylisopropylmethylamine based on the determination of the melting points of the picrolonates. Cannon[7] described a microchemical test with platinic chloride, a second method using a benzol derivative with a melting point of 134° to 135° C., and a third method based on coupling with p-nitrobenzene diazonium chloride. This same author[8] later described a modified technique for amphetamine determination and reported a collaborative study of previously described methods. Illing[9] reported that the distillation of alkaline mixtures of viscera was practically impossible and that when acidified with acetic acid the distillate gave negative results for amphetamine.

These methods for the most part were found wanting, for our observations, from repeated experiments, indicated that the distillation method alone or the tungstate precipitation without distillation gave interfering color.

Our application of distillation and extraction procedures to aqueous solutions of amphetamine sulfate gave quantitative recoveries. This combined method of purification of organs by precipitation with sulfuric and tungstic acid gave clear solutions which filter rapidly. The method will detect and estimate as low an amount of amphetamine as 0.03 mg. in 25 Gm. of tissue. It can be applied to blood and urine as well as to tissues and does not require a blank to be subtracted.

We found the time for quantitative separation from tissues greatly reduced with this method and found it to be a much shorter procedure than the usual modified Stas-Otto method, used routinely in our laboratory.[10] Another salient feature was the elimination of foaming which was encountered in the Gad distillation.

SUMMARY

A new quantitative method for the determination of amphetamine in organs, using sulfuric-tungstic acid filtrate, distilled, is described.

It is shown that quantitative recoveries were obtained from the application of distillation and extraction procedures to aqueous solutions of amphetamine.

This method is applicable to blood and urine as well as to tissues. It will estimate an amount of amphetamine as low as 0.03 mg. in 25 Gm. of tissue.

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A MICROSCREW CLAMP FOR USE WITH MOHR TYPE OF MICROBURETTES

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THE Koch* microburette, in common use for many years, consists of a small capacity burette, 1 to 10 ml., graduated in 0.01 or 0.05 ml., and connected to a reservoir through a glass stopcock. A troublesome feature inherent with any apparatus having glass stopcocks is the gradual accumulation of particles of grease and a film of grease which prevents wetting of the wall of the graduated tube. This results from gradual loosening of the grease by repeated turning of the stopcock. No lubricant, including silicone, overcomes this difficulty. Frequent treatment with cleaning solution is necessary and involves a lot of time and labor. With heparin assays, acid cleaning after every assay is found necessary to give a good meniscus. Another disadvantage is that after several manipulations the accumulation of grease on the margin of the stopcock bore forms a sort of flap valve which makes accurate control impossible. Control of the flow also becomes difficult as the lubricant becomes thinned out and emulsified with consequent sticking.

To obviate these difficulties a microscrew clamp to use with rubber tubing has been designed. The first consideration was to provide a thumbscrew that would work easily yet without play. Second, a shut-off stop was needed to limit the compression of the rubber tubing just enough to stop the flow. Third, an opening stop seemed desirable to permit instant opening to a predetermined degree. This would regulate the maximum rate of outflow from the burette. The main features of the clamp will be described although Fig. 1 is self-explanatory. A photograph of the clamp is shown in Fig. 2.

The screw clamp shown is designed to take a $\frac{5}{16}$ inch outside diameter pure gum tube, but the clamp may be made for any size. The rubber tube is maintained in position by four pins. A U-shaped clamp, shown in the lower part of the front view and in section *A-A*, is fitted over the lower end of the rubber tube in which is inserted the glass delivery tip and holds the latter steady.

The clamp is held together by two threaded rods, an extension of one serving as the stop. The compression bar is slightly rounded on its contact surface. A strong bronze spring forces the compression bar assembly firmly against the lower end of the thumbscrew through a steel ball bearing. Play is thus eliminated and friction in the step bearing minimized. The thumbscrew turns in a threaded bronze bushing, and the large knurled knob provides for fine control

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From the Department of Pharmacology, St. Louis University School of Medicine.
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These microscrew clamps may be obtained from Earl L. Page, Nashville, Ind.
*Koch, F. C.: Two Convenient Forms of Apparatus for Microblood and Microurine Analysis, J. Lab. & Clin. Med. 11: 774, 1926.

The two adjustable stop levers are threaded to fit the shaft of the thumb screw. Any degree of compression on closing is obtainable, and any degree of opening up, to a turn of about 330 degrees. Loosening both levers permits opening to the maximum distance as needed when inserting the rubber tube, or in draining and cleaning the burette. The entire assembly is supported by a 3/8 inch rod clamped to a suitable support. Except for the steel ball bearing, all parts are of nonferrous metal.

The lower end of the microburette has a side outlet for connecting to the reservoir. This outlet is placed 45 degrees to the rear on the right side so that both the microscrew clamp and another smaller screw clamp controlling the reservoir may be operated by the right hand.

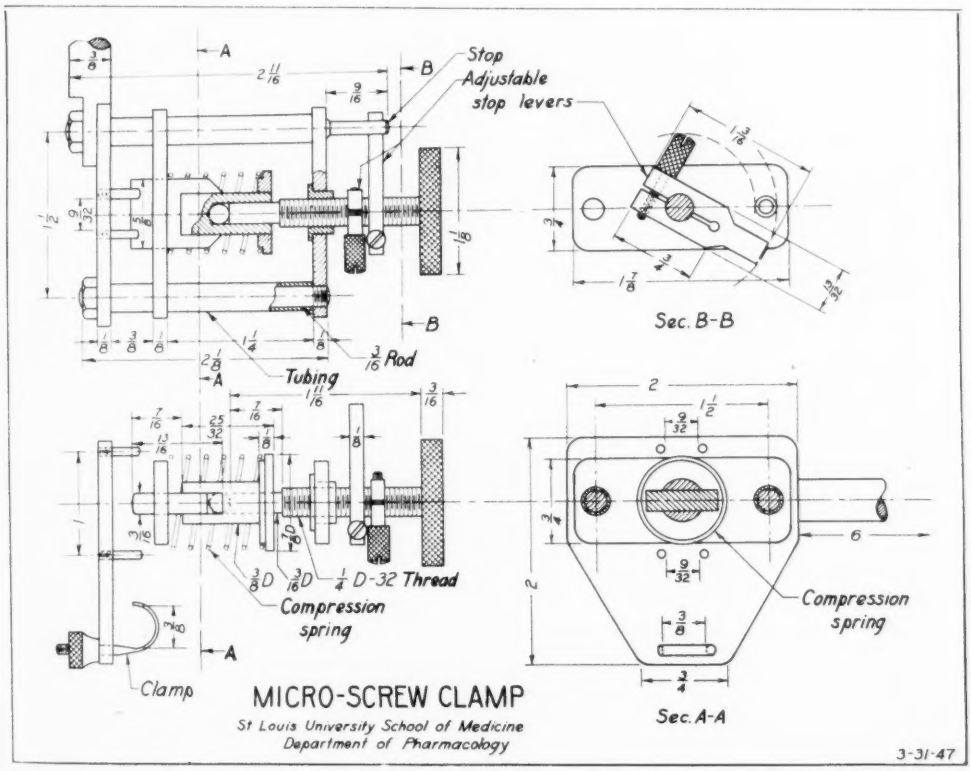

Fig. 1.

The change in liquid level in the burette due to compression of the rubber tubing after closure is reduced to a small and *constant* value by setting the closing stop lever to a point where the flow is just stopped. The volume of the liquid displaced may be as little as 0.001 or 0.002 ml. and depends partly on the quality of the rubber tubing. Skill is quickly acquired in compensating for this when definite volumes are being dispensed. In titrations there is no concern for this since readings are made only after closure.

It has been found that this microscrew clamp offers an ease of manipulation quite outside the range provided by glass stopcocks. In heparin assays several hundred portions of solutions have been dispensed from a series of microburettes. One's fingers become tired in adjusting the glass stopcocks, even to the point of being unable to control accurately a stopcock still turning smoothly. This microscrew clamp virtually eliminated fatigue in the experiments run, and the speed of operation was increased. Volumes as small as 0.001 ml. may be dispensed if the burette bore is small enough. A microburette with this specially designed screw clamp should be useful not only in heparin assays, but also in many types of work where small volumes are to be dispensed, or for microtitrations.

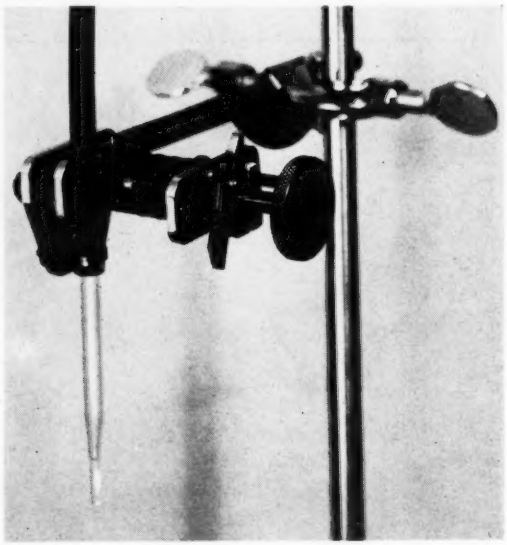

Fig. 2.

SUMMARY

A microscrew clamp has been described for use with microburettes which offers the following advantages over glass stopcocks:

1. Minute control to 0.001 ml. is possible.

2. Faster operation is attained through the use of adjustable stops for the open and closed positions.

3. Faster operation is also attained by the greater ease of manipulation which also reduces fatigue.

4. The frequency of the troublesome acid cleaning is reduced by the elimination of the glass stopcock; and because there is no lubricant to collect on the glass walls, cleaning time itself is reduced.

AN INSTRUMENT FOR RECORDING MULTIPLE PRESSURES IN MAN

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Introduction.—The instrument to be described was developed in order to record multiple pressure changes used in study of the cardiovascular system. These are intra-arterial pressure, pneumatic cuff pulsations and pressure, finger plethysmograph, venous pressure, and respiration. This instrument does not represent an original development as to principle, but it is felt that the modifications and adaptations of previously published techniques have proved to be of sufficient practical value to be described. It was also felt that the method of optical recording with high frequency manometers described by Hamilton and co-workers,[1] and subsequently modified for special purposes by various authors (Green,[2] Gregg and associates,[3] Kotte and associates,[4] and Cahoon and co-workers,[5] was the simplest and most accurate available. However, the relatively long optical levers proved to be too cumbersome for general clinical use in a hospital, where it was desirable to have an instrument that could be transported from one ward to another and used in well-lighted rooms. Portability coupled with the necessity for recording four to six pressures simultaneously required considerable modification of the usual optical manometers.

General Description (Fig. 1).—The present instrument consists of three separate units (Fig. 1). One (*A*, Fig. 1) houses the light source, transformer, manometers and holders, and timing device. It measures 81.28 cm. (32 inches) in length, 38.10 cm. (15 inches) in width, and 35.56 cm. (14 inches) in height. The second unit (*B*, Fig. 1) is the photokymograph or camera, the base of which is 21.59 cm. (8½ inches) square and the height, 30.48 cm. (12 inches). This construction enables the camera to be used for other purposes. The third unit (*CPS*, Fig. 1) contains a compressed air tank, pressure indicators, and control valves for inflation and deflation of a pneumatic cuff.

Optical System (Fig. 2).—The optical system employed resembles in principle those previously described in that it consists of a light source, the beam of which is reflected from a small mirror mounted on an elastic membrane to moving photographic film (Fig. 2). It is so arranged that the optical length from the mirror to the film is 80 centimeters. The light source consists of a battery of inverted "V" filament 6-volt bulbs arranged to give a vertical filament image. The lights are enclosed in a small plywood housing and require no special ventilation. A cylindric lens which corrects the image of the lamp filament in the vertical axis is placed in front of each manometer. Mounted in the camera is a cylindric lens which corrects the image of the lamp filament in a horizontal axis, thus producing a square or rectangular spot on the film.

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†Dr. Edwards died on Jan. 6, 1947.

Timing Device.—The timing device, giving transverse lines at 0.10 second intervals, consists of a lamp with a rotating slotted disk between it and the camera. The disk is driven by a synchronous motor.

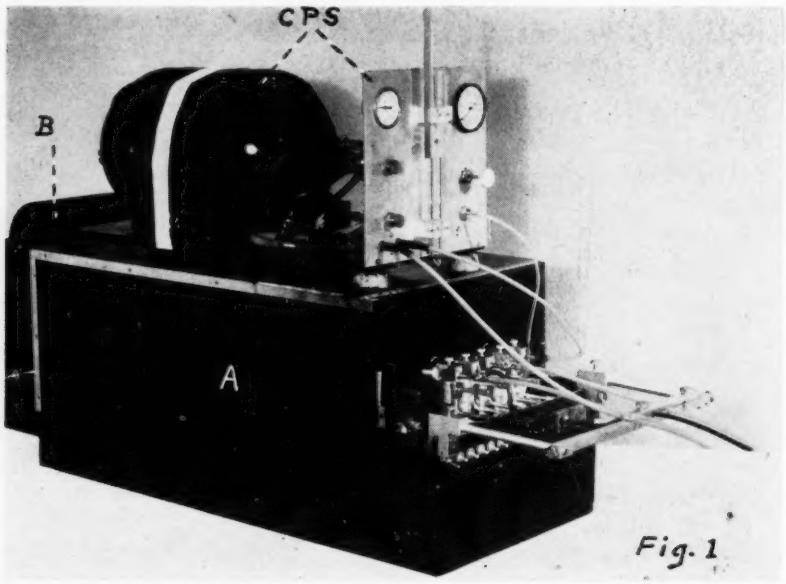


Fig. 1.—General assembly. *A*, Housing for light source, transformer, manometers and holders, and timing device; *B*, camera; *CPS*, compressed air tank, pressure indicators, and control valves for inflation and deflation of cuff.

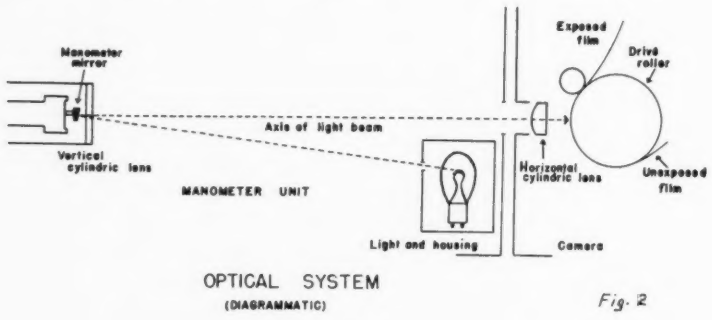


Fig. 2.

Manometers.—The diameter and tension of the manometer membranes vary with the type of pressure to be recorded. Latex rubber is used. For the lowest pressure range (plethysmograph), a large slack manometer membrane (diameter, 2.5 cm.) is used. For intra-arterial pressure recordings, which are in the highest pressure range, tense membrane manometers of 0.5 to 1 cm. in diameter are used. Recently, beryllium copper has become available again and is replacing the latex membranes in higher pressure ranges.

Manometer Holders (Fig. 3).—Each manometer assembly is mounted on a small platform on which it can be rotated around a vertical axis so as to place

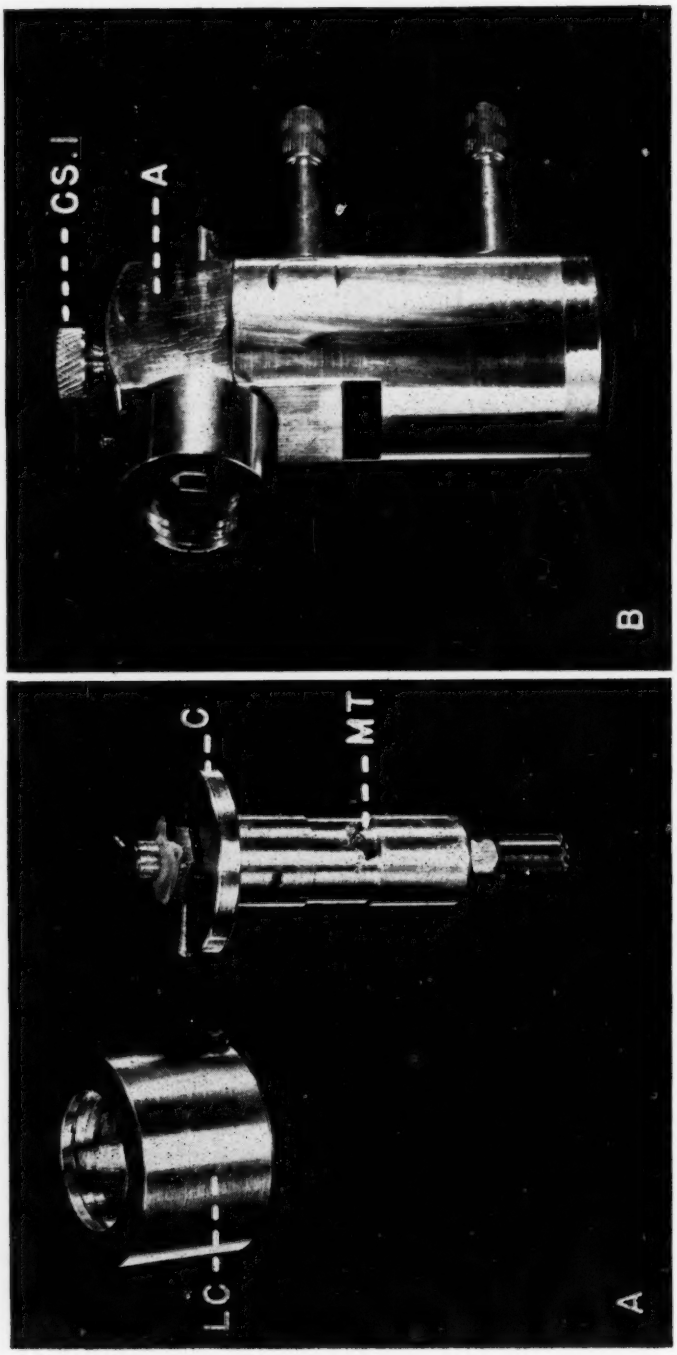
the reflected light beam at any position along the camera slot. This position is maintained by a single clamping screw. The block (*A*, Fig. 3, Part *C*) containing the manometer tube rotates on an axis parallel (around pin *P*, Fig. 3, Part *C*) to the camera slot and may be clamped in position by a single screw. This movement serves to elevate or depress the reflected light beam in relation to the camera slot. The manometer tube is inserted into a hole in this block so that it may be rotated 360 degrees around a horizontal axis perpendicular to the camera slot and may be clamped in any position by a single screw. This allows the axis of deflection of the light beam to be made parallel to the camera slot (horizontal). The assembled manometers are shown in Fig. 3, Part *D*.

Tubing.—The tubing used to connect the intravascular needles with the manometer should be relatively nonexpansile lest serious damping of the system occur. Polyethylene plastic tubing (I.D., ± 4 mm.; wall, ± 2 mm.) has been found to have several distinct advantages over lead, tin, or zinc tubing. It is chemically inert to the solutions that it contains and therefore forms no precipitate, and it is transparent so that air bubbles may be readily observed. Its flexibility makes it easy to handle.

With the advent of small diameter thin-walled polyvinyl tubing which may be passed through 17- or 15-gauge needles, the possibility of utilizing this material and discarding the rigid indwelling needles has been considered. A flexible tube which is relatively inelastic and has a large inside diameter as compared with the outside diameter has many theoretical advantages for intravascular studies. In addition the clotting time of blood in such a tube is greatly prolonged as compared with that in a steel needle tube. For veins, a 17-gauge needle is introduced into the vein and a length of polyvinyl tubing inserted through the needle within the vessel for several centimeters (or any desired length); the needle is then withdrawn, leaving the tubing within the vessel. It is then connected to a manometer by a suitable adapter and flexible tubing. Little bleeding takes place around the indwelling tube.

Finger Plethysmograph.—The usual water-filled glass tube plethysmograph has been replaced by an air-filled thin-walled aluminum tube 12 cm. (4.72 inches) in length and 3.5 cm. (1.38 inches) in diameter, which is equipped with a screw-on collar that allows cones with openings of various diameters to be fastened to the tube. The plethysmograph tube with cone in place is slipped over the finger and one of the quick-drying liquid plastics is applied. By this method only relative changes in volume which take place over short periods of time such as with acute venous occlusion (finger blood flow) may be measured.

Differential Chamber (Fig. 4).—To record the arterial pressure pulses transmitted through a pneumatic cuff, a manometer must be able to withstand cuff pressures up to 300 mm. Hg or more, yet be responsive to pressure changes of 1 to 5 mm. Hg. The differential capsule principle has been utilized and a small chamber which fits in the described manometer holders has been developed. The membrane holder is screwed into the manometer tube in the usual fashion. The lens cap, with the lens sealed in place, is then placed over the membrane holder and screwed to the manometer tube. Thus a pressure-tight chamber is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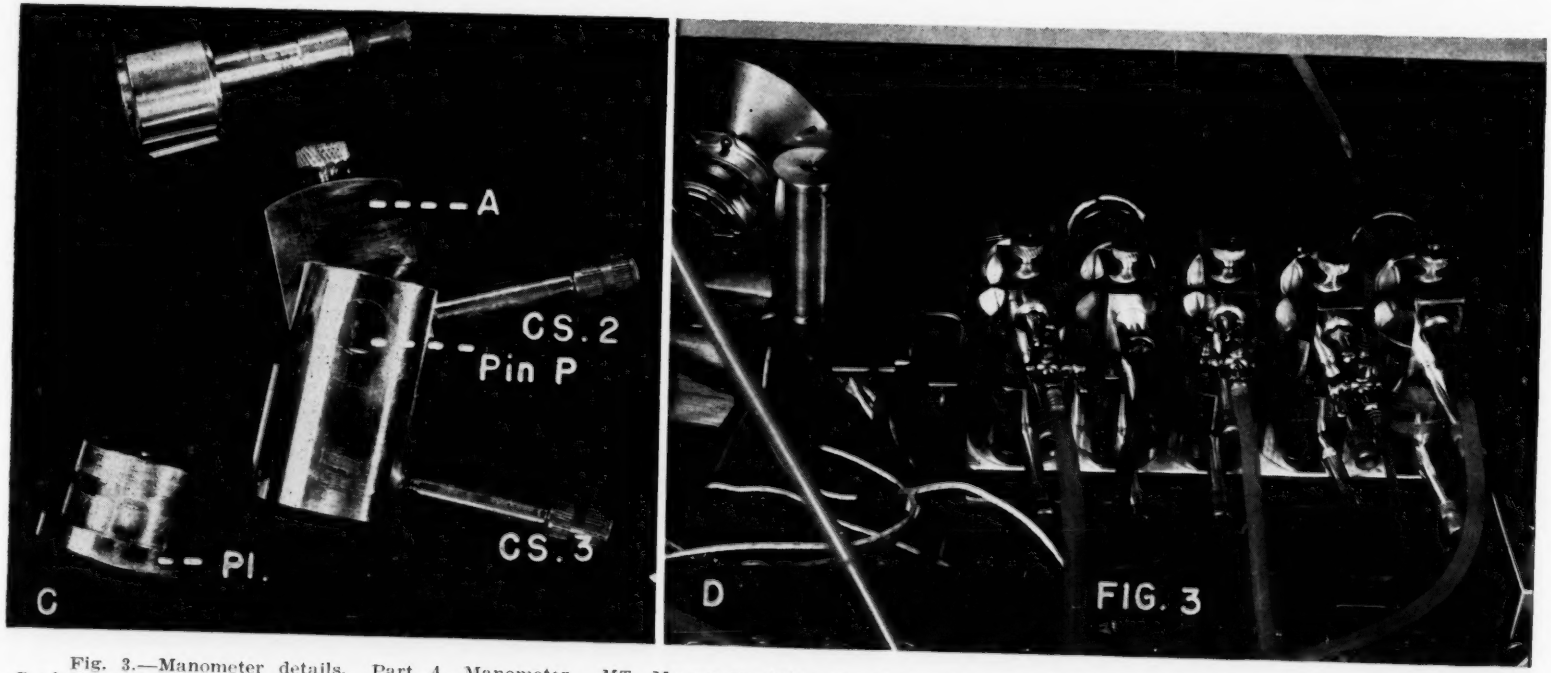

Fig. 3.—Manometer details. Part *A*, Manometer. *MT*, Manometer tube; *C*, collar; *LC*, cap containing cylindric lens which is fitted to collar *C* of the manometer.

Part *B*, Manometer assembly. *A*, Manometer block. This block contains a circular opening into which the manometer tube fits and allows for rotation of the tube. The tube can be fixed in any desired position by tightening of screw, *CS.1*.

Part *C*, Manometer assembly. *A*, Manometer block; *Pl.*, platform; *CS.2*, screw for elevation or depression of light beam; pin *P* which acts as axis for rotation of block *A* (this axis is parallel to the camera slot); *CS.3*, screw for rotation of assembly on platform (the axis for this rotation is at right angles to the camera slot).

Part *D*, Arrangemennt of manometers.

formed around the membrane. An opening into this chamber allows pressure to be applied to the mirror surface of the membrane, while pressure may be applied to the opposite side through the manometer tube. If the two sides are then connected to the cuff, equal pressures will be applied to both sides of the membrane and no movement will take place. However, a small needle valve is placed in the tube leading to the chamber which enables the rate of pressure change to be controlled so that at any pressure level sudden changes in pressure are not transmitted to the chamber and hence act solely on one side of the membrane. The result is the recording of cuff pulsations at any cuff pressure levels desired.

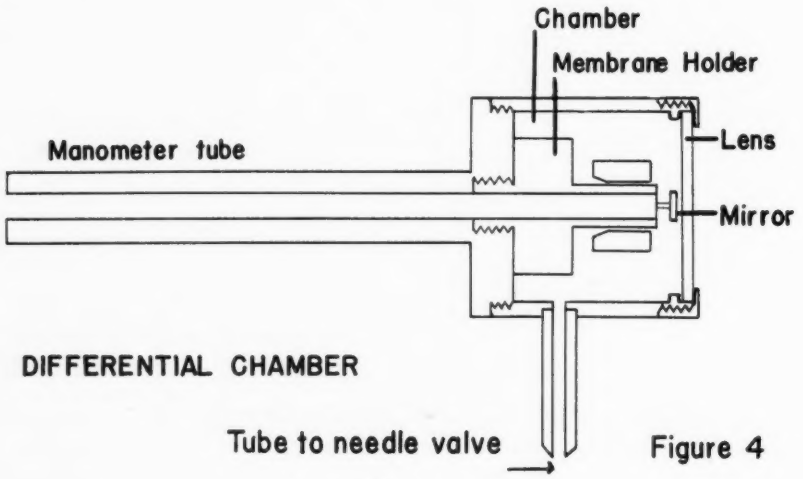

Fig. 4.

Camera.—The camera is of the usual type and uses 6-inch seismographic recording paper.

Records (Fig. 5).—The types of records obtained by the instrument described are illustrated in Fig. 5, Parts *A*, *B*, and *C*. Fig. 5, Part *A*, shows the recording of direct arterial pressure (brachial artery) and indirect arterial pressure from the same arm as the cuff pressure is dropped from above to below the systolic level. The time lines are placed across the entire width of this record. In Part *B*, the time line is placed at the bottom of the record only. This record shows the plethysmographic tracing as well as direct and indirect arterial pressure records and cuff pressure. The recording of venous pressure and respiration is demonstrated in Part *C*. Here the direct and indirect arterial pressure is recorded as the cuff pressure is decreased from above to below diastolic pressure.

SUMMARY

A new instrument is described for simultaneous recording of intraarterial pressure, pneumatic cuff pulsations and pressure, finger plethysmograph, venous pressure, and respiration. It is easily movable and can be used in well-lighted rooms. The principle is similar to that of the Hamilton manometer.

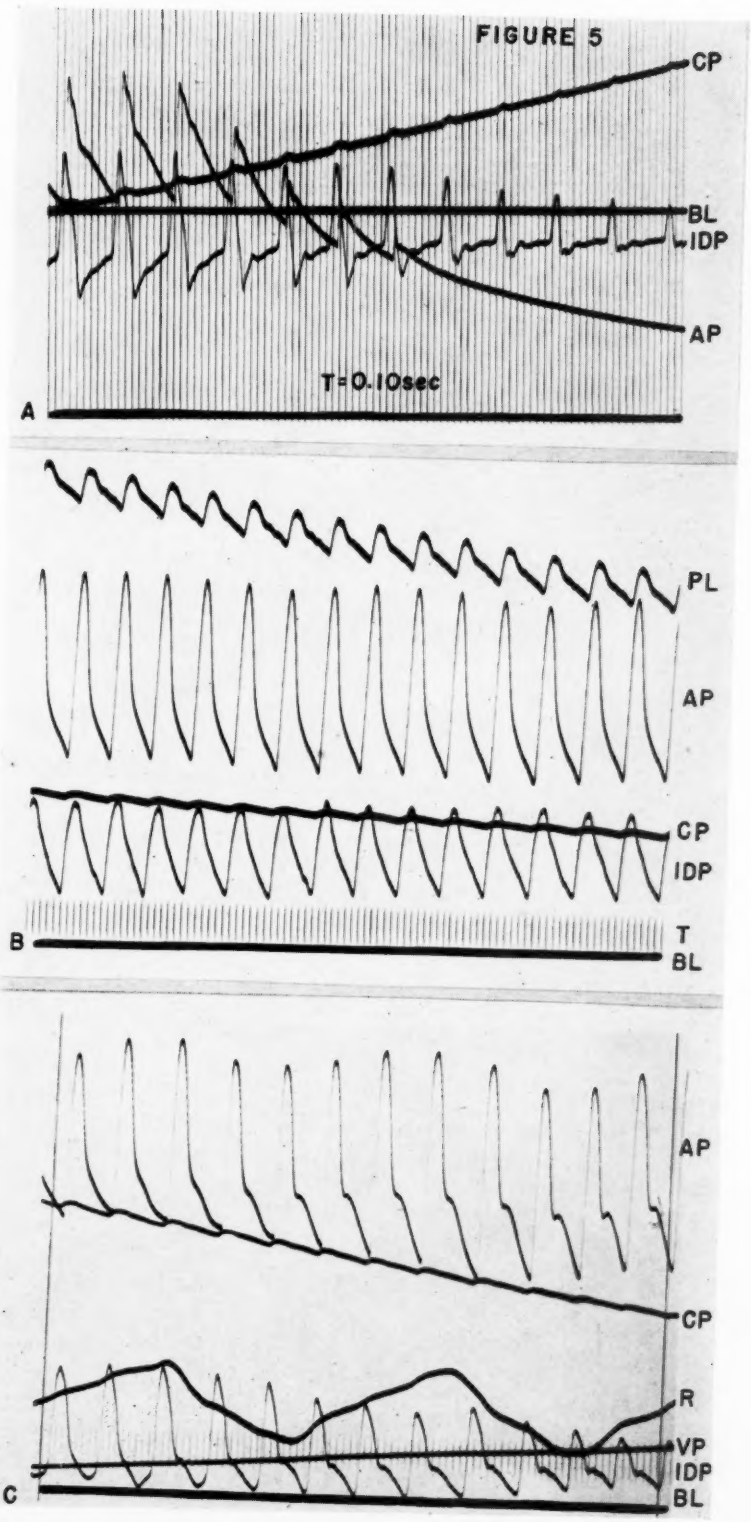

Fig. 5.—Parts *A*, *B*, *C*, Examples of typical records. *CP*, Cuff pressure; *BL*, base line; *AP*, direct arterial pressure; *IDP*, indirect arterial pressure; *PL*, plethysmograph; *R*, respiration; *VP*, venous pressure.

Plastic tubing is substituted for metal tubes and needles previously used. A differential chamber is described for recording pneumatic cuff pulsations.

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